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Does modulation of amylin's second messenger system influence its anorectic effect?

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1 Zusammenfassung

Das Ziel der Studie war zu untersuchen, ob der Phosphodiesterase Inhibitor Bay 73-6691 (Bay), den anorektischen Effekt von Amylin verstärkt. Die Versuche basierten auf der Hypothese, dass die Hemmung der Phosphodiesterase (PDE) und damit eine Verlangsamung des Abbaus von cGMP (dem second messenger von Amylin), die Amylin Wirkung verstärkt.

Unter der Wirkung von Bay kam es allerdings nicht zu einer Verstärkung oder Verlängerung des anorektischen Effekts von Amylin, gleichgültig ob Bay peripher oder zentral appliziert wurde. Stattdessen konnten wir unter einigen Versuchsbedingungen beobachten, dass Bay allein auch bereits zu einer Reduktion der Futteraufnahme führte. Dies könnte bedeuten, dass Bay eine Verstärkung des Effekts von endogenem cGMP auslöste. Im folgenden Experiment konnten wir ausschliessen, dass der Effekt von Bay auf die Futteraufnahme auf der Induktion einer Geschmacksaversion beruhte, d.h. Bay verursachte keine Übelkeit. Letztlich konnten wir zeigen, dass Bay die Amylin-induzierte c-Fos Expression im Gehirn erhöhte.

Mit dieser Studie konnten wir zeigen, dass unter den gewählten Versuchsbedingungen Bay die Wirkung von Amylin nicht zuverlässig verstärkte. Leider waren einige unserer Experimente schwierig zu interpretieren, da wir keinen deutlichen Amylineffekt unter Basisbedingungen hatten. Dies könnte an der hohen Konzentration von DMSO liegen, welches zum Lösen von Bay verwendet wurde und deshalb auch als Kontrollinjektion diente.

2 Summary

The aim of this study was to investigate if Bay 73-6691 (Bay), an inhibitor of phosphodiesterase 9A (PDE), enhances amylin's anorectic action. We hypothesized that inhibition of PDE, resulting in reduced degradation of amylin's second messenger cGMP, would prolong amylin's anorectic action.

Bay appeared to be unable to reliably enhance or prolong amylin's effect on eating under most test conditions, i.e. peripheral or central administration of Bay. Under some conditions, however, Bay treatment reduced food intake, similar to animals receiving amylin. This suggests that Bay might enhance the action of endogenous cGMP. In a subsequent experiment, we ruled out that Bay decreased eating due to the induction of malaise, as Bay did not produce a conditioned taste aversion. Co-treatment of Bay and amylin produced a significant increase in c-Fos signaling in the brain, compared to rats treated with Bay/saline.

With this study we showed that under the conditions used, Bay did not reliably enhance amylin's anorectic effect. Unfortunately, some of our experiments were difficult to interpret because we found no clear effect of amylin on eating under baseline conditions. Whether this was due to an effect of the high concentration of DMSO used to dissolve Bay and which served as control injection in vehicle/amylin treated rats, needs to be elaborated.

3 Introduction

3.1 Obesity problematic

Obesity and the state of being overweight are defined by the World Health Organization (WHO) as an abnormal fat accumulation relative to lean mass, which can lead to health problems (WHO, 2013). To assess a person's body condition, the body mass index (BMI) is usually used; the BMI is the weight of a person divided by the square of his/her height. However this assessment of body condition is sometimes imprecise because it does not differentiate between the lean and the fat mass of a person, and it can also lead to miss interpretation in the elderly (Shah and Braverman, 2012). Nevertheless, the evaluation of BMI is a valuable initial indicator of a a person's body condition because it is easy to determine and because in most adults, changes in body weight are mainly due to changes in fat and not in lean mass. A BMI ≥ 25 is considered as overweight and a BMI ≥ 30 is considered obese.

According to the WHO, worldwide obesity has more than doubled since 1980, and 65 % of people live in a country were obesity and the associated diseases kill more people than underweight. In 2005, it was predicted that by 2030 the number of overweight or obese people would reach 1.35 billion (Kelly et al., 2008). However, these estimations were too conservative, because according to the WHO in 2008 there were already 1.4 billion overweight adults worldwide, and of these, 200 million men and nearly 300 million women were obese. People who suffer from overweight or obesity are at higher risk to develop an illness which is associated with excessive weight, like type 2 diabetes, metabolic syndrome, fatty liver disease and number of cardiovascular diseases (Malnick and Knobler, 2006), and obesity is also associated with higher mortality (Drenick et al., 1980).

The fight against obesity is currently one of the most discussed topics in medicine and a variety of approaches can be used to tackle this growing problem. However, many efforts to treat obesity successfully have failed. Changes in lifestyle, e.g. by consuming fewer calories and burning more calories through increased physical activity, is one of the most obvious and least invasive ways to lose weight. However, since changes in lifestyle are often not sufficient nor sustainable in the longer term, there is a growing demand for alternative and more effective ways to reduce body weight, such as through hormone therapy and surgical

intervention. While many mono- and combination hormonal therapies have been developed, and often demonstrated promising effects on weight loss initially, most of these treatments do not sustain the weight loss in the long term. Alternatively, several types of bariatric surgery are available, like gastric banding, sleeve gastrectomy and the Roux-en-Y gastric bypass (RYGB); these invasive procedures reportedly have high success rates and seem to be the only effective treatments that also lower obesity related mortality and its comorbidities. For example, in one study patients undergoing RYGB surgery maintained a loss of 27.7% of the initial body weight 6 years after surgery (Adams et al., 2012). However, bariatric surgery is invasive, expensive, and reserved for patients who are severely obese. Thus, there is an unceasing need for the development of new therapeutic strategies. Ongoing efforts are targeting the different peripheral and central mechanisms which mediate satiation, satiety, hunger and, therefore, also weight gain and weight loss; however these circuits are not yet fully understood. It is only when our understanding of these mechanisms increases, that the development of new effective anti-obesity treatments with minimal side effects will be possible.

3.2 Physiological control of food intake

Maintenance of body weight homeostasis requires a control of the balance between food intake and energy expenditure. Even if daily food intake varies markedly depending on emotional and social influences, consumption over a longer time period is more tightly controlled. The major controls are mediated by hormonal signals, which are typically gut-derived. Two groups can be distinguished: the short term satiation signals, which are secreted during a meal, and the adiposity signals, which are more tonically secreted, depending on the body's energy stores.

Short-term satiation signals are released primarily from the gut during a meal. The first satiation signal discovered was cholecystokinin (CCK), which is secreted by the I cells of the duodenal and jejunal mucosa following the ingestion of food (Buffa et al., 1976). Fat, protein and amino acids are highly potent stimulators of CCK release, whereas glucose does not increase CCK plasma levels as effectively (Liddle et al., 1985). Treatment with exogenous

CCK dose-dependently reduces food intake in rats (Gibbs et al., 1997), and it is thought that these actions are mediated by CCK1 receptors localized mainly on vagal afferents, but perhaps also in the hypothalamus and in the brainstem (Blevins et al., 2000). Additional examples of short-acting satiation signals are glucagon-like peptide 1 (GLP-1) and peptide YY (PYY). Both hormones are released from the L-cells of the ileum and colon, and act via vagal afferents but, depending on the route of administration, perhaps also act centrally to inhibit food intake.

The second category, referred to as adiposity signals, are typically secreted in proportion to the amount of fat mass, and influence body weight and food intake via access to the central nervous system (Woods et al., 1998). This group includes the pancreatic hormones insulin and amylin (Morley and Flood, 1991), as well as leptin, which is produced by adipocytes (Considine et al., 1996; Maffei et al., 1995). Circulating levels of both leptin and insulin correlate to the amount of fat mass (Considine et al., 1996) and when injected in the brain cause body weight loss and a reduction in food intake (Woods and Seeley, 2000). However, circulating levels of leptin and insulin also fluctuate acutely in response to fasting or eating, similar to satiation hormones.

3.2.1 Hypothalamus-hindbrain axis

The hypothalamic-hindbrain axis plays a vital role in processing hormonal information and integrating signals from peripheral tissues like the pancreas, the gut and white adipose tissue in order to maintain energy balance. The hypothalamus is located in the mediobasal part of the brain, and is composed of different nuclei that control various aspects of energy homeostasis, including the ventromedial nucleus (VMH), the lateral hypothalamic area (LHA), the paraventricular nucleus (PVH), and the arcuate nucleus (ARH). In addition to these nuclei, hindbrain structures, including the nucleus of the solitary tract (NTS) and the area postrema (AP), are also key players in the control of energy balance. Peripheral information has different ways of reaching these central sites. Some information is mediated through the vagus nerve or the spinal cord via vagal or splanchnic afferents. Aside from a few exceptions, the brain is protected by the blood brain barrier (BBB), which functions to separate the blood from the brain's extracellular fluid, even though some hormones, like amylin, are able to

cross the BBB (Banks et al., 1995). Another route of access is via regions of the brain that lack a fully functional BBB, which are called circumventricular organs (CVO). Hormones from the blood have direct access to these sites. One example of a CVO is the AP, which plays an important role in the hypothalamic-hindbrain axis.

Each of the aforementioned brain nuclei consists of distinct neuronal populations that release a variety of neuropeptides or neurotransmitters, which contribute to various aspects of energy homeostasis. Located at the base of the hypothalamus, the arcuate nucleus integrates different hormonal and neural signals. The ARH harbors two main neuronal populations that each play an important role in the control of the food intake; one population promotes anabolic actions, while the other mediates catabolic signals (Haskell-Luevano et al., 1999). The catabolic system is mainly mediated by proopiomelanocortin (POMC), which is synthesized in a subpopulation of ARH neurons. POMC is cleaved to produce melanocyte-stimulating hormone (α-MSH), a hormone that acts via melanocortin receptors 3 and 4 in the hypothalamus and other areas to produce a strong anorexigenic effect (Cone, 2005). The anabolic effect is mediated through neurons that synthesize neuropeptide Y (NPY) and agouti-related peptide (AgRP). An increase in these two signals results in a greater drive to eat. This is mediated via activation of the Y receptors and by inhibiting the melancortin 3 and 4 receptor, respectively (Broberger et al., 1998; Cone, 2005; Haskell-Luevano et al., 1999). Both insulin and leptin can act directly on ARH neurons to inhibit AgRP/NPY expression and to stimulate POMC expression (Cone, 2005). Lying just dorsal to the ARH, is the VMH. While the exact phenotype of VMH neurons controlling food intake is unclear, lesions to the VMH demonstrably lead to hyperphagia and therefore also obesity (Nakagawa et al., 2000). Another study shows that electrical stimulation leads to the opposite effect, such that food deprived animals will eat less following stimulation of the VMH (Anand and Dua, 1955). While not proven, there is evidence that the VMH is a potential site of interaction for amylin and leptin (see below). In an attempt to better define the role of the VMH, Elmquist and colleagues searched the brain systematically for the mRNA of the long form of the leptin receptor and found out that these are localized in specific nuclear groups in the brain. The VMH, together with the DMH, expresses high concentrations of leptin receptor mRNA. In the VMH the mRNA is located to the dorsomedial division (Elmquist et al., 1998). Hence, leptin can act directly in the VMH, and after administration of leptin c-Fos activation can be seen in

a very similar distribution to the leptin receptor mRNA (Elmquist et al., 1998). If and how amylin interacts with leptin in the VMH is yet to be elucidated.

3.3 Amylin

Amylin is a 37 amino acid peptide that is co-secreted with insulin and synthesized by pancreatic β cells in response to nutrient stimuli (Cooper et al., 1987; Ogawa et al., 1990). During and shortly after food intake, the plasma concentration of both hormones increases. Amylin's primary function is to complement the action of insulin to stabilize glucose homeostasis after food intake. Amylin accomplishes this by inhibiting glucagon secretion and slowing gastric emptying during and after a meal (Gedulin et al., 2006; Young, 1997). In addition to these glucoregulatory effects, amylin also dose-dependently reduces food intake after intraperitoneal injection (Lutz et al., 1995). Furthermore, if infused into the hepatic portal vein at meal onset, amylin inhibits feeding within minutes by reducing meal size and duration, but without having an effect on the following intermeal interval (Lutz et al., 1995). Injection of amylin directly into the brain also reduces food intake, while the infusion of the amylin antagonist AC 187 can block this effect when given in conjunction with amylin, or promote food intake when administered alone (Mollet et al., 2004). It seems that amylin's anorectic effect is specific and not caused by general malaise in the rat, since amylin does not elicit a conditioned taste aversion (Morley et al., 1997).

There is strong evidence that peripheral amylin's anorectic effect depends on activation of neurons located in the AP of the hindbrain. Lesions of the AP lead to the loss of amylin's anorectic ability (Lutz et al., 1998), and abolished amylin's effect on gastric emptying (Edwards GL, 1998). The AP expresses a high density of amylin receptors (Sexton et al., 1994), which are G-coupled protein receptors comprising a core calcitonin receptor (CT-R) and receptor modifying activity proteins 1 or 3 (RAMPs; Muff et al., 1999). The occurrence of amylin receptors in other brain areas, such as the nucleus accumbens, the VMH, and the NTS, has been described (Sexton et al., 1994), but their function still remains to be elucidated in respect to the actions of peripheral amylin.

C-Fos is a nuclear protein often used as a marker for neuronal activity. In response to extracellular signals, this transcription factor is upregulated and exerts either inhibitory or stimulatory effects on gene transcription (Curran and Morgan, 1995). When amylin is administered in a dose sufficient to reduce food intake, c-Fos can be detected immunohistochemically in different regions of the brain, including the AP, the NTS, the external lateral parabrachial nucleus (LPBE), the central nucleus of the amygdala (CEA), and the lateral division of the bed nucleus of the stria terminalis (BSTL; Riediger et al., 2001; Riediger et al., 2004; Rowland et al., 1997). In this manner, the neuronal activation in response to amylin can be quantified; importantly, neurons that may be inhibited by amylin cannot be detected with the c-Fos technique directly in response to amylin injection. Additionally, electrophysiological studies have shown that amylin application directly excites AP neurons, and that this effect can be blocked by the amylin antagonist, AC 178 (Riediger et al., 2001).

3.4 cGMP, phosphodiesterases and phosphodiesterase inhibitors

Following the binding to its receptor, amylin induces cGMP formation in AP neurons (Riediger et al., 2001). cGMP acts as a second messenger in the area postrema and is thought to mediate the anorectic response of amylin. This is supported by electrophysiological data where the membrane-permeating analog 8-bromo-cGMP, which mimics cGMP accumulation, stimulated 65% of all area postrema neurons and 7 out of 9 amylin sensitive neurons (Riediger et al., 2001). Additionally, it was shown that direct infusion of 8-bromo-cGMP leads to a food intake reduction by reducing meal size, exactly like amylin (Mollet et al., 2004). Mechanistically amylin's second messenger cGMP might affect other second messenger systems, like cAMP, through the opening of ion-channels or affecting the activity of phosphodiesterases in the cytoplasm (Schmidt et al., 1993).

One important regulator of cGMP activity is a group of enzymes called phosphodiesterases (PDEs). PDEs are molecules that breakdown intracellular cyclic nucleotides like cGMP and cAMP, and came to the general attention as targets for drug development for a wide range of different diseases. There are 11 known different PDE families, which each have different

splice variants and isoforms. A single cell type can express various different PDE types, which have the task to degrade cyclic nucleotides in order to prevent them from accumulating and spreading to other areas of the cell. PDEs can be regulated through different mechanisms, including stimulation by the Ca^{2+}/calmodulin complex, phosphorylation, and dephosphorylation (Bentley, 2005). Allosteric binding to their substrates and protein-protein interaction can also regulate the PDE function.

In 1967, caffeine was one of the first described inhibitors of PDEs (Cheung, 1967), and since then, many PDE inhibitors have been developed to treat a variety of diseases. One of the most famous indications is the PDE5 inhibitor sildenafil (Viagra ®), which is used to treat erectile dysfunction but also e.g. pulmonary hypertension. Sildenafil blocks PDE5 from degrading nitric oxide-induced cGMP produced in the smooth muscle cells lining the blood vessels of the corpus cavernosum. The prolonged action of cGMP causes the muscle to relax and increases blood flow to the penis, resulting in a more potent and longer erection (Boolell et al., 1996; Rajfer et al., 1992). While this is just one example, the same concept is being used to target other PDEs which are localized to various tissues in the body.

3.5 Phosphodiesterase 9A and its inhibitor Bay 73-6691

Phosphodiesterase 9A (PDE9A) specifically hydrolyzes cGMP with the highest known affinity of any PDE (Fisher et al., 1998; Guipponi et al., 1998). Twenty-one different splice variants of PDE9 have been discovered (Rentero et al., 2003), and while the physiological relevance of this diversity is not completely understood, it is suggested that the isoforms differ in intracellular targeting, biochemical properties and other characteristics (Beavo, 1995).

Analysis of mRNA expression in the rat brain has shown that PDE9A is distributed in the basal forebrain, the olfactory bulb, and various cortical and hippocampal regions (Andreeva et al., 2001). It seems that the pattern of central expression of PDE9A closely resembles the pattern of the soluble guanylate cyclase, suggesting a cooperation of these two enzymes in order to precisely control cGMP levels (Andreeva et al., 2001; Matsuoka et al., 1992). More recently and in collaboration with the laboratory of Steven Smith (Orlando), we have shown

that PDE9A is also expressed in the AP; this was shown using transcriptome analysis of that rat hindbrain (unpublished results). Due to the presence of PDE9A in the AP, and the fact the amylin treatment induces a cGMP response in this same region, it seems plausible that PDE9A may influence amylin action through the degradation of its second messenger cGMP.

Indeed, sparse, but encouraging, evidence identified PDE9 inhibition as a potential therapeutic target for treating diseases from Alzheimer's to diabetes. The nonselective, competitive PDE inhibitor IBMX is not able to inhibit PDE9A, nor are several other PDE inhibitors including vinpocetine, rolipram, tadalafil (Wunder et al., 2005). Fortunately, several potent and selective inhibitors of PDE9 have been developed in recent years (Deninno et al., 2009; Kleiman et al., 2012), including the commercially available Bay 73-6691. Bay 73-6691 is a novel inhibitor, which inhibits specifically the PDE9A with only moderate inhibition of the other PDEs (Wunder et al., 2005), and which is thought to have a half-life of approximately 2 hours in vitro (van der Staay et al., 2008). Currently Bay 73-6691 is under preclinical development against Alzheimer's disease and also it was shown that it improves learning and memory in rats (van der Staay et al., 2008; Wunder et al., 2005).

More recently, Kleiman and colleagues demonstrated, that blockade of PDE9A, by a variety of PDE9A inhibitors leads to a dose-dependent accumulation of cGMP. Since cGMP is part of multiple downstream neurotransmitter systems, blocking of the degradation by PDE9A might yield therapeutic benefits especially in the field of psychiatric and neurodegenerative diseases. The findings of Kleiman show that PDE9 inhibition can reverse disruptions of working memory by ketamine, or reverse the stereotypic scratching response to mescaline. (Kleiman et al., 2012). Furthermore, DeNinno and colleagues described a PDE9-knock out mouse which showed reduced weight gain when placed on a HFD, in addition to reduced insulin resistance and a lower fat mass (Deninno et al., 2009). These findings show the potential importance of the PDE9 modulation for therapeutic benefits.

3.6 Can Bay 73-6691 enhance amylin's anorectic action?

The first half of this dissertation will focus on the potential modulation of amylin action by PDE9 inhibition. As previously discussed, amylin induces cGMP formation in the AP (Riediger et al., 2001). According to our hypothesis, cGMP is degraded by PDE9A, resulting in the termination of amylin's anorectic effect (Figure 3.1). Hence, we postulated that treatment with the PDE9 inhibitor, Bay 73-6691, would slow down the breakdown of cGMP, and therefore enhance and prolong amylin's anorectic action (Figure 3.2). To test this hypothesis, several trials were conducted to test how pretreatment with Bay 73-6691, administered intraperitoneally, subcutaneously, or directly into the 4th cerebral ventricle, alters the feeding response after saline or amylin injection.

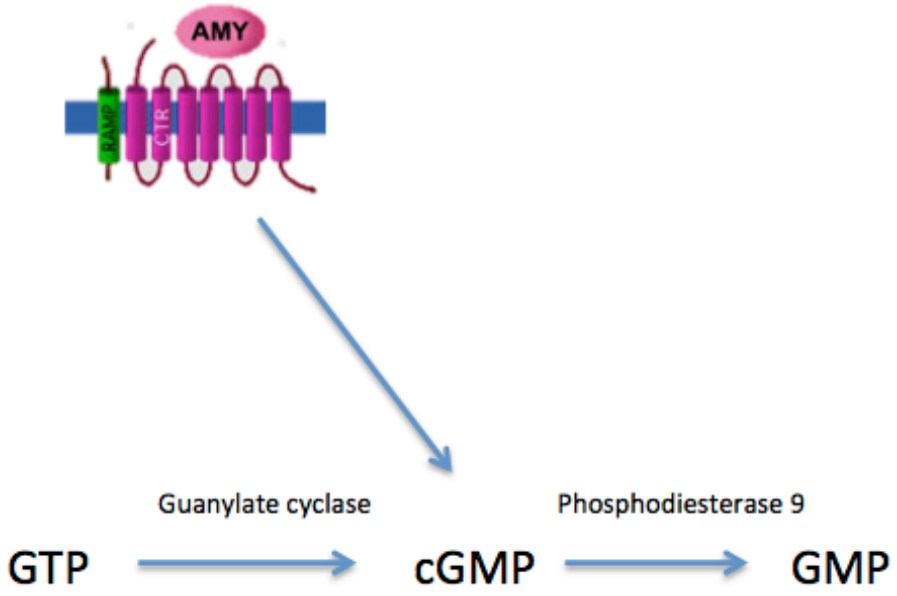

Figure 3.1: Working model for the mediation of amylin's anorectic action: amylin induces cGMP, which is broken down by phosphodiesterase 9 to terminate amylin action.

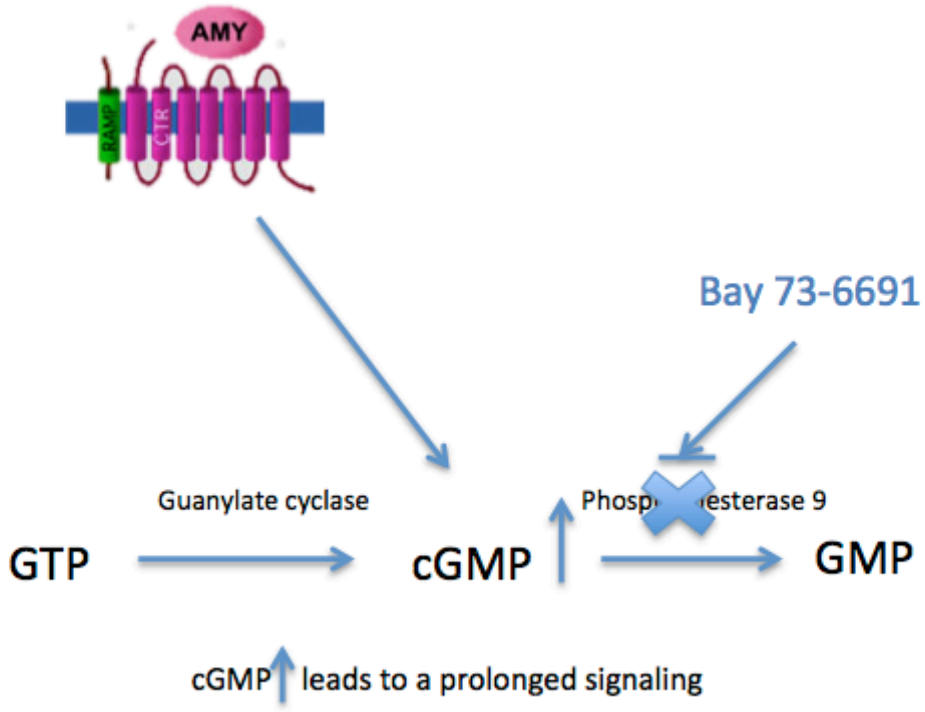

Figure 3.2: Working model for the mediation of amylin's anorectic action: if the degradation of cGMP were prevented by the PDE9 inhibitor, Bay 73-6691, cGMP would be expected to accumulate and amylin signaling may be prolonged.

3.7 The role of the AP in amylin-induced restoration of leptin sensitivity

The second half of the dissertation focuses on whether amylin action in the AP is necessary to observe the synergy between amylin and leptin, which is described in detail below.

3.7.1 Leptin

Leptin is a product of the obese gene (ob) secreted by adipocytes and was first discovered in 1994 (Zhang et al., 1994). When chronically administered in leptin sensitive individuals either centrally or peripherally, leptin reduces body weight through a reduction in food intake and an increase in energy expenditure (Halaas et al., 1995; Widdowson et al., 1997). The leptin receptor (LepR) is part of the interleukin-6-receptor family (Tartaglia et al., 1995). There are multiple isoforms of the LepR, which are produced from a single Lepr gene, and can be

divided into three classes: secreted, long and short (Tartaglia, 1997). To produce its effects on body weight, leptin binds to the long form of the leptin receptor (LepRb), which results in signal transduction via the noncovalently associated tyrosine kinase of the Jak kinase 2 family. The activation of Jak2 then phosphorylates and activates signal transducers and activators of transcriptions (STATs), in this case STAT3 (Baumann et al., 1996; Ghilardi et al., 1996; Ihle and Kerr, 1995). The phosphorylation of STAT3 (pSTAT3) is, therefore, often used as a marker of LepRb activation and leptin sensitivity. Based on the localization of LepRb and the expression pattern of pSTAT3 following leptin treatment, it is generally assumed that the ARH is a primary central target of leptin action (Baskin et al., 1999; Elmquist et al., 1998; Hubschle et al., 2001). Other nuclei thought to mediate leptin action include the VMH, the dorsomedial nucleus of the hypothalamus, the ventral premamillary nuclei of the hypothalamus, the ventral tegmental area, the lateral parabrachial nucleus, and probably also the NTS.

3.7.2 Leptin resistance in the obese state

Since its discovery leptin has been studied intensively for its potential as an obesity treatment. While leptin reduces body weight in obese, congenitally leptin deficient individuals or rodents (Halaas et al., 1995; Licinio et al., 2004), it is typically ineffective in promoting weight loss in obese phenotypes that have no defect in leptin production (Knight et al., 2010; Zhang and Scarpace, 2006)}. The reason is that obese humans and rodents typically exhibit elevated circulating leptin levels, a condition known as hyperleptinemia; unfortunately, this chronic increase in leptin concentration cannot prevent overeating and obesity, rather the body responds by becoming leptin resistant. Therefore, in most cases of human and animal obesity, leptin has little effect, and on its own is often ineffective to treat obesity (Halaas et al., 1995; Lin et al., 2000).

While the mechanism underlying leptin resistance is not yet clear, there are several proposed causes. One possibility is that leptin transport across the BBB is saturated and leptin no longer reaches its receptors in the brain (Banks and Farrell, 2003; Caro et al., 1996). A second mechanism may be related to the overstimulation of the LepRb, which then responds with

negative feedback signaling (Knight et al., 2010). In support of this theory, increased levels of the suppressor of cytokine signaling-3 (SOCS-3), which blocks leptin signaling, have been detected in the ARH of leptin-resistant, obese mice (Munzberg et al., 2004). Furthermore, heterozygous SOCS-3-deficient mice show greater sensitivity to leptin, with enhanced STAT3 signaling, increased weight loss, and protection against diet-induced obesity (DIO) (Howard et al., 2004). These results suggest that SOCS-3 might play an important role in leptin resistance and, more generally, in the control of leptin sensitivity.

3.7.3 Amylin-Leptin interactions and the role of the AP

Several lines of evidence demonstrate that combined treatment of amylin and leptin has beneficial effects; the most notably being amylin-induced restoration of leptin responsiveness in obese humans and rats (Roth et al., 2008). The combination treatment lead to a greater body weight loss and inhibition of food intake than would be predicted by mere addition of the effects of the individual monotherapies; thus suggesting that amylin and leptin work synergistically to produce these effects. This combination treatment also lead to increased energy expenditure, where fat was the primary substrate used, rather than carbohydrates (Trevaskis et al., 2008). Roth also compared a pair-fed group with the leptin/amylin-treated group and could therefore show that the greater efficacy was not only due to the decreased food intake elicited by amylin, but that the presence of amylin was actually required for the synergistic effect with leptin (Roth et al., 2008) Interestingly, the effect seemed to be specific to amylin, as combining leptin with other satiating hormones, like PYY or GLP-1, did not produce the same level of fat specific weight loss (Roth et al., 2008). To maintain this weight loss, vehicle or leptin treatment seemed to be insufficient, and only further amylin/leptin treatment prevented rats from regaining their bodyweight (Trevaskis et al., 2010).

In addition to these effects in rats, a similar synergistic phenomenon was also observed in human subjects. Pramlintide, an amylin analogue, produced significant body weight loss and food intake reduction when administered alone (Hollander et al., 2004; Smith et al., 2007), but the combination treatment of pramlintine and metreleptin was even more powerful (Roth et al., 2010).

How the interaction of these two hormones is mediated is not yet clear. There is currently no evidence that one neuronal population mediates the convergence of these two hormones, and it seems more likely that the synergy occurs via an activation of complementary signaling pathways. Several studies point towards the VMH as playing a pivotal role in this interaction. Most notably, Roth and colleagues showed that amylin can restore leptin responsiveness in DIO rats, as further indicated by an increase in pSTAT3 signaling in the VMH (Roth et al., 2008). While amylin did not restore leptin-induced pSTAT3 in the ARH of DIO rats, chronic, peripheral infusion of the two agents lead to an amplified pSTAT3 response in the ARH of lean rodents (Turek et al., 2010). Additionally, receptor autoradiography showed that leptin binding in the VMH is increased by amylin (Turek et al., 2010), indicating that the VMH might play an important role for the convergence of these two satiation hormones.

The AP is an unlikely primary site of converge for the two hormones, since injections of leptin did not enhance the amylin induced c-Fos signaling in this brain region (Turek et al., 2010). However, it is not clear if activation of the AP by amylin is required for the amylin/leptin synergy to manifest, or if amylin interacts with leptin via an alternate pathway. In order to determine the role of AP in the leptin/amylin interaction, we attempted to repeat the study of Roth and colleagues in DIO rats and extended the study by using rats with or without a lesion of the AP.

4 Material und Methods

4.1 Animals and housing conditions

Male Wistar rats or male Sprague Dawley rats (Elevage Janvier, Le Genest Saint Isle, France) were used for all experiments. Upon arrival in our facility, the rats weighed between 225 and 250 grams. The rats were maintained in a temperature-controlled environment (21 ± 2°C) on a 12h/12h light-dark cycle. Water and food were accessible ad libitum, unless otherwise described. The animals were handled on a daily basis for the first 4 weeks, following which they were handled every 1-2 days. Throughout the course of an experiment, bodyweight was measured every 2-3 days. Animals were habituated to the housing conditions for at least 7 days prior to any experimental manipulation. All experiments were approved by the Veterinary Office of the Canton of Zürich, Switzerland.

4.1.1 Housing conditions in macrolon cages

Animals were group-housed in macrolon cages (2-5 rats/cage) prior to undergoing surgery or the start of a feeding or immunohistochemical experiment.

4.1.2 Housing conditions in hanging wire mesh-cages

For most experiments, animals were single-housed in wire mesh cages; two different sizes were available and used according to the rats' body weight:
23cm x 39cm x 20cm for rats <400g
47cm x 33cm x 20cm for rats >400g
Food hoppers were mounted externally to facilitate the manual measurement of food intake.

4.1.3 Housing conditions in the BioDAQ system

For experiments requiring continuous measurement of food intake, animals were single-housed in plexiglass Tecniplast cages (Type IV S; 48cm x 38cm x 21cm) fitted with the BioDAQ Food Intake Monitoring system (Research Diet Inc., New Brunswick, NJ, USA). Environmental temperature and light intensity were measured 24 h per day by the system. The BioDAQ system converts the animals' feeding behavior into data which can be analyzed and

stored. Data collected from individual food hoppers were analyzed using the BioDAQ Data Viewer, which provides the data in raw format or as graphs and tables. Food hoppers are attached to the outside of the cage and rest on a strain gauge that measure the weight of the hopper each second; weight changes of the hopper during and after an interaction with an animal were converted to eating bouts. If bouts occured one after another within the timeframe of the user-specified intermeal interval (15 min), bouts were clustered into meals. If the hopper weight remained stable for more than 15 min, the meal was considered terminated. These data from the food hopper were permanently stored for further analysis. The data could then be analyzed according to the time of the day, the light cycle and affiliation to a group. Hence, this system allowed the continuous assessment of total food intake, size and number of meals and meal pattern in the dark and light phase, respectively.

4.2 Experimental diet

For most experiments, rats were maintained on a standard rodent chow (Diet 3430; Kliba Nafag Provimi Kliba AG, Kaiseraugst, CH).
Major Nutrients (% per weight):

Dry Matter	88%
Crude protein	18.5%
Crude fat	4.5%
Crude fiber	4.5%
Crude ash	6.3%
NfE	54.2%
Gross Energy	16.1 MJ/kg
Metabol. Energy	13.2 MJ/kg
Starch	35.0 %

Table 4.1.1: Standard Chow from Kliba Nafag

In some cases, rats were maintained on a high fat diet (#D12266; Research diets Inc., New Brunswick, NJ, USA).

D12266 Formula:

Product	**Kcal%**
Protein	16.8
Carbohydrates	51.4
Fat	31.8

Table 4.1.2: High fat diet from Research diets

4.3 Peptides and Drugs

Amylin (Bachem AG, Bubendorf CH, or Amylin Pharmaceuticals Inc, San Diego, CA, USA) was diluted in 0.9% NaCl (Fresenius Kabi AG, Stans, CH) at various concentrations. Leptin (Peprotech, Rocky Hill, New Yersey, USA) was diluted at various concentrations in aqua ad injectabilia. CCK-8 sulfated (Bachem) was prepared with 0.9% NaCl to a concentration of of 3 μg/ml. All peptides were prepared immediately before the start of the experiment and were kept cold on ice until injection.

For central injection studies the PDE9 inhibitor Bay 73-6691 (Sigma Aldrich, St Louis, MO, USA), was diluted in 75% Dimethyl sulfoxide (DMSO; Fluka, Sigma Aldrich) and 25% aqua ad injectabilia, and was sonicated for 15 min to promote dissolving (Branson 1200, Bender +Hobein, Zürich). When Bay 73-6691 was used for peripheral injection, it was diluted in 20% DMSO and 80% aqua ad injectabilia and then treated as described above. 5-Thio-D Glucose (Carbosynth) was diluted in 0.9% NaCl to reach a concentration of 210 μg/3 μl.

4.4 Surgical techniques

4.4.1 Cannulation of the 4th cerebral ventricle

Rats underwent 4th ventricle cannulation under isoflourane anesthesia (Abbott AG, 6341 Baar). Anesthesia was induced with 5% isofluorane and was lowered to a maintenance level of 2.5% during surgery. One day prior to surgery, rats were treated with Enrofloxacin (Baytril ®; s.c. 10mg/kg BW; Bayer, Provet SA 3421 Lyssach), and were treated post-operatively with Flunixin-meglumin (Flunixin; s.c. 2.5mg/kg BW of 1:50 dilution; Dr. E Graeub AG, CH-3018 Bern), Baytril, Prednisolon (Prednisolut ®; s.c. 2mg/kg BW; Mibe GmBH 06796 Brehna), and NaCl (Fresenius Kabi). The antibiotic and the analgesic treatment continued until 3 days post-operation.

For implantation of guide cannulas, rats were secured in the stereotactic frame (Stoelting Lab Standard Stereotaxic Instrument, Stoelting, IL, USA), and the scalp was shaved and sanitized with Braunol. A 2 cm-long incision was made at the midline from cranial to caudal in order to visualize Lambda and Bregma. A flat-skull position was achieved by measuring the dorsoventral coordinates of Lambda and Bregma, following which the site of cannulation was calculated based on the coordinates of Bregma (See Table 4.1.3). Then, a hole for the 4th ventricle cannulation was drilled. The coordinates for the 4th ventricle were obtained from the stereotaxic brain atlas of Paxinos & Watson (Paxinos and Watson, 1998). Three additional holes were made for screws (Stainless steel mounting screws 0-80 x 1/8, length 3.2mm) in a radius of 5 mm around the cannulation hole. The screws were implanted as anchor points for the dental cement (Technovit 4071; H. Kulzer & Co. GmbH Wehrheim Taunus,BRD) to secure the pedestal of the cannula. The guide cannula (22 G C313G/Spc, cut 9mm below ped; Plastics One, Roanoke, VA) was lowered to the targeted site, optimally 1.0 mm above the 4th ventricle, and was then fixed with dental cement. After the drying of the dental cement, a cannula dummy (cannula dummy implant C315DC/SPC, 9mm no projection; Plastics One) was inserted, and the skin was sutured with Vicryl 4-0 (Johnson+Johnson Intl, Belgium), if necessary. Rats received analgesics and antibiotics for 3 days post-surgery. The coordinates for the two cannulation experiments are described with the individual experiments.

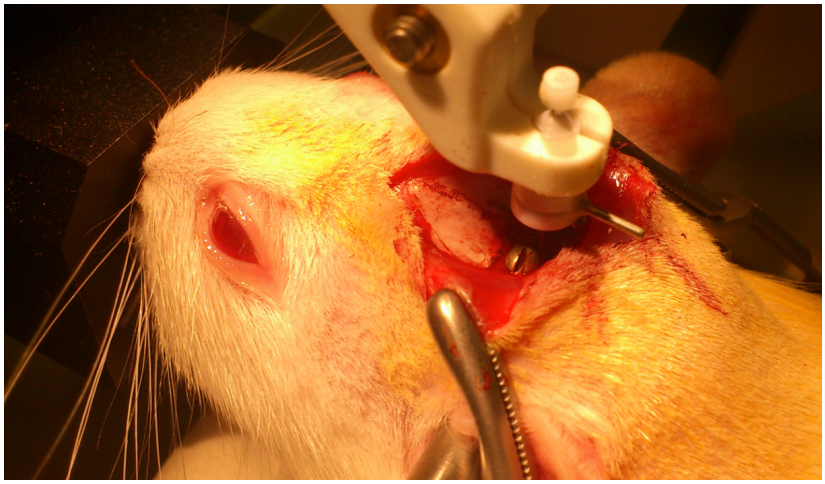

Figure 4.1: Cannulation of the 4th ventricle

Example for calculated implantation position:

Source	Anterior -Posterior	Dorsal-Ventral
Coordinates of Bregma (β)	32.0	47.0
Distance from β to 4th ventricle in atlas	-11.6	-6.6
Coordinates for implanting:	**20.4**	**40.4**

Table 4.1.3: Example calculation for cannulation coordinates

4.4.1.1 5-Thio-D-Glucose Test

The 5-thio-D-glucose (5TG) test was performed to verify correct placement of the 4th ventricular cannula. Animals were fasted for 3 h before dark onset, and the test began 2 hours before dark onset. Baseline blood glucose was determined for each rat and then 5TG (210 µg/3 µl) was administered slowly in the 4th ventricle by inserting an internal cannula that extended 1.5 mm from the guide cannula. Blood glucose was again measured 30 and 60 min following the infusion of 5TG. Food was given back at the end of the test. If blood glucose increased by a minimum 4.5 mmol/l above baseline, the animal was considered positive and correct cannula placement was confirmed (Potes et al., 2012). If a rat was not deemed positive

with the 1.5 mm internal cannula, the test was repeated with a 2.0 mm internal cannula. The same criteria were applied.

4.4.1.2 Central Injections

For the 4th ventricular injections Silastic Tubing (ID 0.762 x OD 1.651mm; Tagelswangen, Switzerland) was attached to a Hamilton syringe (10 μl, Hamilton Bonaduz AG, Bonaduz, CH) and filled with the relevant drug solution, with special care to avoid any air bubbles. Prior to filling, the tubing was marked for the volume, which should be delivered (3 μl). The free end of the tubing was then attached to the internal cannula (C313I/Spc internal cannula fits 9mm guide with 1.5 mm or 2.0 mm proj. and a beveled tip; Plastics One). The dummy caps covering the guide cannulas were removed and the internal cannula carefully inserted. A volume of 3 μl was then slowly and carefully infused. After the volume was delivered, the internal cannula was left in place for 1-2 min before removal, to prevent backflow of the infusate out of the cannula.

4.4.2 Area postrema lesion

Animals underwent area postrema lesion (APX) surgery under a mixture of ketamine (100mg/kg; Narketan, Vetoquinol) and xylazine (5mg/kg; Streuli Ag, Uznach) anesthesia. Vitamin A ointment (Bausch & Laub Swiss AG) was applied generously to the eyes to prevent dry eyes. The scalp was shaved and sanitized with Braunol. Animals were placed in a stereotactic frame, and fixation of the head was made with the help of the ear bars and the nosepiece, which pressed the head down in order to visualize the crista occipitalis. A 1.5 cm long vertical incision with the scalpel was made from 0.5 mm above to 1 cm below the crista occipitalis and the first layer of muscles were cut. The second and third layer of muscles were bluntly dissected in order to visualize the atlanto-occipital membrane. A T-shaped cut was made directly under the crista occipitalis in the dura membrane, and if the membrane was obstructing the view, it was carefully removed with a bent needle. The area postrema was then aspirated by vacuum sucking using a blunted 26 G needle. After verifying the absence of the area postrema visually, the wound was sutured with Vicryl 4-0. Flunixin, prednisolone and saline were administered post-operatively as indicated above. Post-surgical treatment with painkillers and antibiotics continued for three days or longer if indicated. The protocol

for the APX was modified from the thermal area postream lesions performed by Mc Glone and colleaques (McGlone et al., 1980).

4.5 Conditioned Taste Aversion

Wistar rats were subjected to the conditioned taste aversion protocol outlined below, based on the paradigm used by le Roux (le Roux et al., 2011). Rats were fluid deprived for 19.25 hours followed by 30 min access to water or saccharine (0.3%); saccharine was given only the first 30 min of fluid access on conditioning days, while water was given in all other occasions. After an additional fluid deprivation of 3.5 h rats had again access to water for 45 min. Following saccharine access on conditioning days (day 6, 9 and 12), rats received one of the following intraperitonal injections: vehicle, Bay 73-6691 (0.1mg/kg) or lithium chloride. On experimental day 14, rats were given access to both water and saccharine for 30 minutes and the respective amounts consumed were recorded.

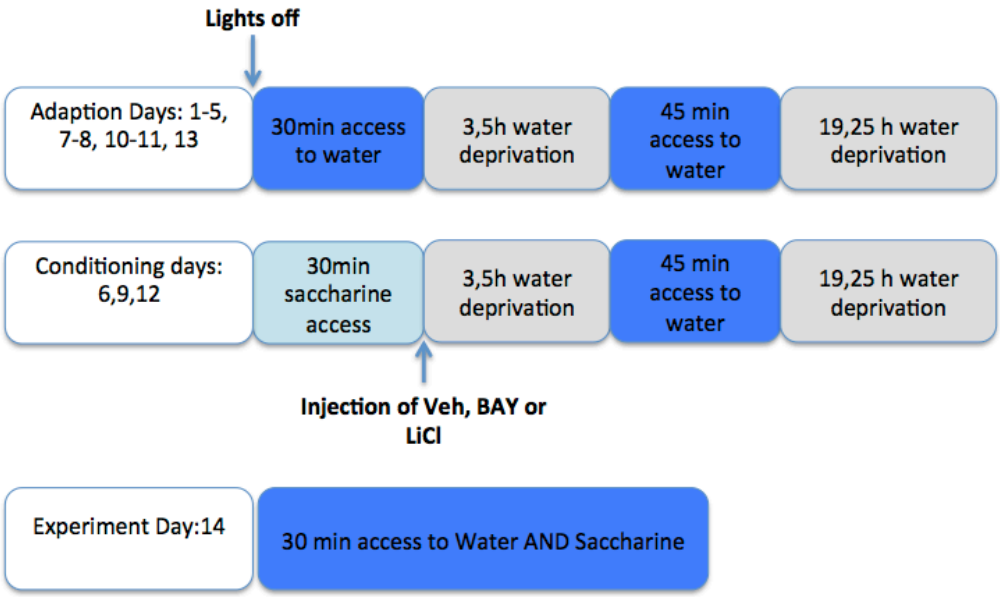

Figure 4.2: Conditioned Taste Aversion schedule

4.6 Implantation of minipumps

On the day of implantation, minipumps (Alzet, osmotic minipumps model 2002, Charles River; Wilmington MA, USA) were weighed and the empty weight was noted. Half of the minipumps were filled with the experimental peptide or drug solution, and the other half filled with the appropriate vehicle. To fill the pumps, a 1-ml syringe (Omnifix, Braun; Melsungen, GE) was fixed to an injector, which was delivered together with the minipumps. After the filling the weight of the pump was measured again to verify that the pump was correctly and completely filled (mean filling volume: 213 μl). Prior to implantation, the pumps were placed in a container of sterile saline. If the minipump was attached to a saline filled tube, priming was needed, which was done by placing the minipump in a container with sterile, 37°C-warm saline. In this environment, which resembles the body fluid environment and temperature after implantation, the pump started delivering the drug, replacing the saline in the tube with the drug solution. After the priming the pump could be used directly, delivering the desired substance immidiately after implantation.

4.7 Perfusions protocol and Immunohistochemistry

Two perfusion protocols were used for detecting c-Fos or pSTAT3, respectively, in rat brain tissue.

4.7.1 Perfusion protocol for detection of neuronal marker c-Fos

Animals were injected with pentobarbital (60mg/kg; Kantonsapotheke Zürich) and perfused intracardially (100 ml/min) with 0.1 M phosphate buffer (PB) solution for 90 seconds, followed by ice cold 4% paraformaldehyde (Sigma- Aldrich, pH 7.2) in 0.1 M PB for 120 seconds. After this procedure, the brains were removed and post-fixed in 4% paraformaldehyde solution for 2 hours, followed by 48 hours cryoprotection in 20% sucrose/PB solution.

<u>0.1 M Sodium phosphate buffer (PB) preparation</u>**<u>:</u>**
3000 ml dH20

32.8 g Sodium phosphate dibasic dehydrate (Sigma Aldrich Steinheim)
12.68 g Sodium phosphate monobasic monohydrate (Sigma Aldrich Steinheim)
Adjust pH to 7.4 with NaOH

4% Paraformaldehyde solution:
1000 ml dH_20
80 g Paraformaldehyde (Sigma Aldrich Schnelldorf, Germany)
1000 ml 0.2 M PB
Drops of NaOH
Adjust pH to 7.2 with Hcl

4.7.2 Perfusion for detection of neuronal marker p-STAT3

The animals were injected with pentobarbital as indicated above and perfused intracardially (100 ml/min) with 0.9% NaCl solution for 90 seconds, followed by ice cold 2% paraformaldehyde (Sigma-Aldrich, pH 7.2) in potassium-phosphate-buffered saline (KBPS) for 120 seconds. After this procedure the brains were removed and post-fixed in 2% paraformaldehyde solution for 2 hours, followed by 48 hours cryoprotection in 20% Sucrose/KBPS solution.

KPBS (0.02 M):
9.7 g KH_2PO_4
56.6 g K_2HPO_4
178.2g NaCl

2% Paraformaldehyde solution:
1000 ml dH_20
40 g Paraformaldehyde (Sigma Aldrich Schnelldorf, Germany)
1000 ml 0.2 M PB
Drops of NaOH
Adjust pH to 7.2

4.7.3 Brain tissue processing

The brains were frozen in chilled hexane (Fluka Chemie GmbH, Buchs SG, CH) on dry ice, and stored at -20°C until sectioned coronally using a cryostat (Leica CM3050S, Nussloch, Germany). Twenty-micrometer sections collected from the hindbrain (containing AP and NTS; according to Paxinos and Watson, 2007) were thaw mounted onto adhesion glass slides (Superfrost Plus, Gerhard Menzel GmbH, Braunschweig, Germany).

If brain sections were stained using free-floating immunohistochemistry, sections were cut 30 μm thick and stored in cryoprotectant at -20°C until staining.

Cryoprotectant:
50% 0.1M PB
30% ethylene glycol
20% glycerol

4.7.4 Gelatinizing Slides

Solution:
1.5 g Gelatin, type A, 220 or 275 Bloom
0.25 g Chromium potassium sulfate
500 ml Distilled water

Water was heated to 60°C to dissolve all substances, then glass slides (Thermo scientific, Menzel Gläser geschnitten) were dipped 3-4 times in the gelatinizing solution and left to air dry.

4.7.5 c-Fos Immunohistochemistry

The c-Fos immunohistochemistry was performed in free-floating brain sections. Sections were washed 2 x 5 min with 0.1 M PB to rinse off cryoprotectant. Sections were incubated in 0.5% H_2O_2 in dH_2O for 10 min to quench endogenous peroxidase, followed by 3 x 10 min

washing in 0.1 M PB. Sections were blocked for 1 h in 0.1 M PB containing 0.3% Triton X-100 (PBTX) and 1% normal goat serum (NGS), followed by an overnight incubation in polyclonal rabbit anti-c-Fos (1:10,000 in 0.3% PBTX and 1% NGS; AB-5, Oncogene from Calbiochem) at room temperature. Sections were washed three times in 0.1% PBTX, and were then incubated for 1 h in biotinylated goat-anti-rabbit secondary antibody (1:400 in 0.3% PBTX; Jackson ImmunoReaseach, Inc). Sections were washed 3 x 10 min in 0.1% PBTX and then incubated for 1 h in avidin-biotin-peroxidase complex (1:500 in 0.3% PBTX; Vectastain ABC kit, Vector). After additional washing steps (2 x 10 min) in 0.1% PBTX, sections were incubated in 3,3'-Diaminobenzidine Tetrahydrochloride (DAB, Sigma) solution for 3 to 4 min to visualize c-Fos-positive cells. The DAB solution was prepared as follows: 2.5 mg DAB per 10 ml 0.1 M PB and 50 μl H_20_2. Sections were then rinsed, mounted on gelatinized slides, dehydrated through an ascending series of alcohols, cleared with xylol, and coverslipped.

4.7.6 pSTAT3 Immunohistochemistry

Slides were first dried for one hour at room temperature, then rinsed in 0.02 M KPBS (2 x 5 min). All rinsing steps were done in 0.02 M KPBS. For demasking, the slides were incubated 20 min at room temperature in 0.3% NaOH (Sigma Aldrich 5M) and 0.3% H_2O_2 (Sigma Aldrich, Steinheim) in KPBS. Demasking is necessary for making a masked substance capable of undergoing reactions. Slides were again rinsed in KPBS (8 x 5 min), followed by a 10 min incubation with 0.3% Glycine (Fluka/Sigma Aldrich Chemie, Buchs, Switzerland) in KPBS. The slides were rinsed before being demasked a second time for 10 min in a 0.03% sodium lauryl sulfate (Carl Roth GmBH, Karslruhe, Germany) in KPBS solution. To block unspecific antibody binding, slides were incubated for 20 min in a blocking solution of 4% normal donkey serum (NDS; Jackson Immuno Research), 0.4% Triton X 100 (Sigma Aldrich), and 1% bovine serum albumin in KPBS. Following a washing step, the slides were then incubated with the primary antibody (1:500, Phospho-Stat3 XP^{TM} Rabbit mAb #9145, Cell Signaling Technology Inc., Denver MA, US) in 1% NDS, 0.4% Triton, 1% BSA in KPBS for 48 h at 4 °C. After washing (8 x 5 min), slides were incubated with the secondary antibody (1:100, donkey anti-Rabbit Alexa-555 IgG (H+L), Invitrogen AG, Basel, CH) in 1% NDS and 0.3 % triton in KPBS for 120 min at room temperature. After a final washing step (8 x 5 min)

the slides were covered with Citifluor (Glycerol/PBS solution Citifluor Ltd., London) and cover slipped.

4.8 Description of individual experiments

4.8.1 Effect of central administration of Bay 73-6691 on the action of peripheral amylin

4.8.1.1 Experiment 1a

Bolus central infusion of Bay 73-6691 with 10 µg/3 µl

Wistar rats (n=36, starting body weight of 225-250 grams; Janvier) underwent 4th ventricular cannulation surgery as described above. The following stereotaxic coordinates were used: anterior-posterior -11.6 dorsal-ventral -6.6, on the midline (Brain Atlas Paxinos & Watson 1998). Rats were allowed to recover for 7 days, after which correct cannula placement was tested using the 5TG test. Twenty-one animals had an increase of blood glucose higher than 4.5 mmol and were therefore included in the study.

On test days, food was removed 2.5 hours prior to dark onset, and 1.5 hours prior to dark onset 3 µl of vehicle or Bay 73-6691 (10 µg) was slowly infused in the 4th ventricle. Saline or amylin (5 µg/kg) was given intraperitoneally 15 min before dark onset. Food was returned at dark onset and the food intake was measured manually at 1, 2, 4 and 24 hours. The study was performed as a four-time crossover so that each animal was tested under each condition (veh/NaCl; Bay 73-6691/NaCl; veh/amylin; Bay 73-6691/amylin).

4.8.1.2 Experiment 1b

Bolus central infusion of Bay 73-6691 with 20 µg/3 µl

The study design matched that described above in experiment 1a, but the dose of Bay 73-6691 was increased to 20 µg Bay 73-6691 dissolved in 3 µl vehicle. A two-time crossover was

performed so that each animal was tested under two conditions. One group received veh/NaCl and veh/amylin, while the other group received Bay73-6691/amylin and Bay73-6691/NaCl.

4.8.1.3 Experiment 1c

Bolus central infusion of Bay 73-6691 with a 3 hours delay

To test an alternate time window of Bay 73-6691 action, experiment 1b was repeated, however the time interval between the Bay 73-6691 infusion and the amylin injection was increased. Food was removed 4 h prior to dark onset, and vehicle or Bay 73-6691 (20 μg/3 μl) was slowly infused into the 4th ventricle 3 h before dark onset. Saline or amylin (5 μg/kg) was given intraperitoneally 15 min before dark onset, and food intake was measured as described above.

4.8.1.4 Experiment 1d

Effect of chronic central infusion of Bay 73-6691 on amylin action

Due to inconlusive results and the assumption that this strain of Wistar rats showed only low sensitivity to amylin, we used Sprague Dawley rats (n=30, starting BW 225- 250 grams; Janvier), which underwent 4th ventricular cannulation surgery as described above. The following stereotaxic coordinates were used: anterior-posterior -11.6 dorsal-ventral -7.2, on the midline (Brain Atlas Paxinos & Watson 1998). Rats were allowed 7 days to recover, after which correct cannula placement was tested using the 5TG test. Twenty-two animals had an increase of blood glucose higher than 4.5 mmol and were therefore included in the study. Coordinates differed from those used in Experiments 1a-d, because this experiment required a different guide cannula (Guide Combination for rats 326OPG/SP Plastic Ones, Op Connector), to which a minipump could be attached.

Eleven animals received a minipump filled with Bay 73-6691, which was placed subcutaneously between the scapulae and connected by a vinyl catheter tubing (Durect, Product od Durect Corp, Cupertina CA) to the guide cannula. The pumping rate was 6.66 μg/h which was delivered directly into the 4th ventricle. Nine animals received minipumps

infusing vehicle. After recovery, rats were transferred to the BioDAQ System in order to continually monitor food intake. Following acclimatization to the new environment, a two-times crossover was performed. On test days, animals were fasted for 2.5 h prior to dark onset, saline or amylin (5 μg/kg; Amylin Pharmaceuticals) were given subcutaneously 15 min prior to dark onset, and then access to food was allowed. Food intake was then continuously measured by the system.

4.8.1.5 Experiment 1e

Activation of c-Fos following chronic central infusion of BAY 73-6691

In a final experiment, rats used in experiment 1d were food deprived for 2.5 h and shortly before dark onset either vehicle or amylin (5 μg/kg) was injected. Food was not given back and animals were perfused after 1.5 h according to the c-Fos perfusion protocol described above. Brains were processed for free floating c-Fos staining.

4.8.2 Effect of peripheral administration of Bay 73-6691 on amylin action

4.8.2.1 Experiment 2a

Peripheral administration of Bay 73-6691

Wistar rats (n=20 starting body weight of 225-250 grams; Janvier) were fasted 2.5 h before dark onset and food was weighed. Bay 73-6691 (1 mg/kg; i.p.) or vehicle (80% aqua injectabila and 20% DMSO) was injected 1.5 hours prior to dark onset. Saline or amylin (5 μg/kg; i.p.) was given 15 min before dark onset. Food was returned at dark onset and the food intake was measured manually at 1, 2, 4 and 24 hours. The study was performed as a four-time crossover.

Due to inconclusive results the experiment was repeated with different parameters: the rat strain was changed to Sprague Dawley, amylin was used from another source (Amylin Pharmaceuticals) and was administered s.c., the amount of DMSO in the vehicle solution was

decreased to 10% and the fasting time was prolonged to six hours. Despite changing these different factors, the outcome of the experiment remained unchanged.

4.8.2.2 Experiment 2b

Conditioned Taste Aversion (CTA)

Wistar rats (n=20, Janvier) underwent a conditioned taste aversion test to determine if Bay 73-6691 induces malaise that may lead to a reduction in eating and hence could mask an effect of the PDE inhibitor to increase amylin's anorectic effect. The 14-day protocol outlined above was used. Rats were subjected to conditioning days, in which they had only limited access to water (1 x 30 min; 1 x 45 min); the rest of the time they were water deprived. On conditioning days, rats had first 30 min access to saccharine followed by an injection of either vehicle, Bay 73-6691 or lithium chloride which was used as a positive control (Nachman and Ashe, 1973), followed by 45 min access to water. On the final test day, rats were given the choice to drink water or saccharine.

4.8.2.3 Experiment 2c

Sensitivity to amylin as assessed by c-Fos activation

This study was performed to determine the rat's sensitivity to amylin, as the results of experiment 2a suggested a reduced sensitivity of the rat strain used here. Therefore 20 Wistar rats from Janvier were fasted for 2.5 h, and 15 min before dark onset the animals received 5 µg/kg amylin (n=5), 20 µg/kg amylin (n=5), 50 µg/kg (n=4), CCK 3 µg/kg (n=1) or Saline (n=5). Food was not given back after injection and after 1.5 h, animals were perfused according to the c-Fos perfusion protocol above. Sections of brains were stained for c-Fos to determine the extent of neuronal activation following amylin administration. CCK, known to produce a robust c-Fos response in the AP and NTS, was used as a positive control. From each rat, five brain sections from the same anatomical level containing the AP/NTS were photographed using the Zeiss AX10Imager.Z2 microscope. The number of c-fos positive cells was estimated in a semiquantitative way.

Some of the experiments yielded unexpected results and therefore different attempts were made to improve amylin sensitivity: the strain of rats was changed, route of administration and also the source of amylin. However, as long as Bay 73-6691 or its vehicle was administered before amylin, no effect of amylin on eating was observed.
Hence, on two occasions, amylin action was tested in the absence of Bay 73-6691 or its vehicle. Rats were fasted for 2.5 h, and 15 min before dark onset amylin was injected (5 μg/kg; i.p.), and food intake was measured 1, 2, and 4 hours later.

4.8.3 The role of the AP in the amylin-induced restoration of leptin sensitivity

4.8.3.1 Experiment 3a

Area postrema lesion and area postrema lesion verification test

Sprague Dawley rats (n=50, Charles River Laboratories International Inc. Wilmington US) were given 2 weeks ad libitum access to high fat diet (HFD) and, based on the percentage of weight gain on this diet, rats were then separated into diet induced obesity (DIO; n=33) and diet resistant (DR) groups. Weight gain over 30 % of the original BW was considered DIO, DR rats gained less than 30%. The DIO rats were maintained on the HFD for a total of 8 weeks, and were transitioned back to standard chow at the time of minipump implantation (see below). The DIO rats were then randomly assigned to the area postrema lesion (APX; n=14) or the sham operated group (n=11). Following recovery, an area postrema lesion verification test was performed. Rats were fasted for 2 hours, and immediately before dark onset, either saline or amylin (5 μg/kg) was injected. Food was given back at dark onset and food intake measured at 1,2 h and 4 h time points. Animals with complete area postrema lesions should not eat less after administration of amylin, and rats showing a reduction in food intake of 15% or greater were omitted from the study because their lesion was considered incomplete. The test was performed as a two-time crossover. Correct lesions of the area postrema were confirmed by this functional test (n=11), and again at the end of the experiment through histology.

4.8.3.2 Experiment 3b

Effect of chronic peripheral infusion of amylin on body weight

Alzet minipumps were purchased from Charles River and filled according to the manufacture's protocol; the pumps were filled with amylin (100 μg/kg/day) or with saline. The four groups of animals were as follows: sham + saline minipump (n=6); sham + amylin minipump (n=7); APX + saline minipump (n=4); APX + amylin minipump (n=6). The minipumps delivered infusates for approximately 14 days.

4.8.3.3 Experiment 3c

Effect of amylin infusion on leptin-induced anorexia

After approximately one week of amylin or saline infusion, leptin-induced anorexia was assessed in DIO rats with or without an AP lesion. Food was removed 2 h before dark onset and either vehicle or leptin (5 mg/kg; i.p.) was injected. Food was given back at dark onset and the amount consumed was measured at 4 h and 24 h post-injection. The trial was designed as a two-time crossover.

4.8.3.4 Experiment 3d

Effect of amylin infusion on leptin-induced STAT3 phosphorylation

The role of the AP in the amylin-induced restoration of leptin sensitivity was again tested in a terminal experiment, in which the leptin-induced phosphorylation of STAT3 was determined in sham or AP-lesioned DIO rats. Thus, on day 13 following the minipump implantation the final trial was performed as follows: Food was removed 2 h before dark onset and at dark onset the animals received either vehicle or leptin (5 mg/kg). Forty-five min after the injections the perfusion was performed according to the pSTAT3 perfusion protocol described above. Brains were then cut and stained for pSTAT3. From each rat, five brain sections from approximately the same anatomical level containing the VMH were photographed using the Zeiss AX10Imager.Z2 microscope. To quantify the number of pSTAT3-positive cells, the

grayscale images were inverted using Adobe Photoshop, positive cells were manually counted, and the average across the five sections was calculated.

4.9 Statistics

All data are presented as mean ± SEM, and were considered significant with a p-value of <0.05. Statistical analyses were done with the program Prism version 5.0 for Mac OS X (Graph Pad Software Inc. San Diego, USA). Crossover data was anlyzed using a two-way Anova with Bonferroni post-hoc test. When only two groups were compared, a Student's t-test was used for analysis.

5 Results

5.1 Effect of central administration of Bay 73-6691 on the action of peripheral amylin

5.1.1 Results of Experiment 1a

Bolus central infusion of Bay 73-6691 with 10 µg/3 µl

To test if Bay 73-6691 enhances the eating inhibitory effect of amylin, rats were pretreated with 10 µg Bay 73-6691 injected into the 4th ventricle, followed by peripheral treatment of amylin just before dark onset

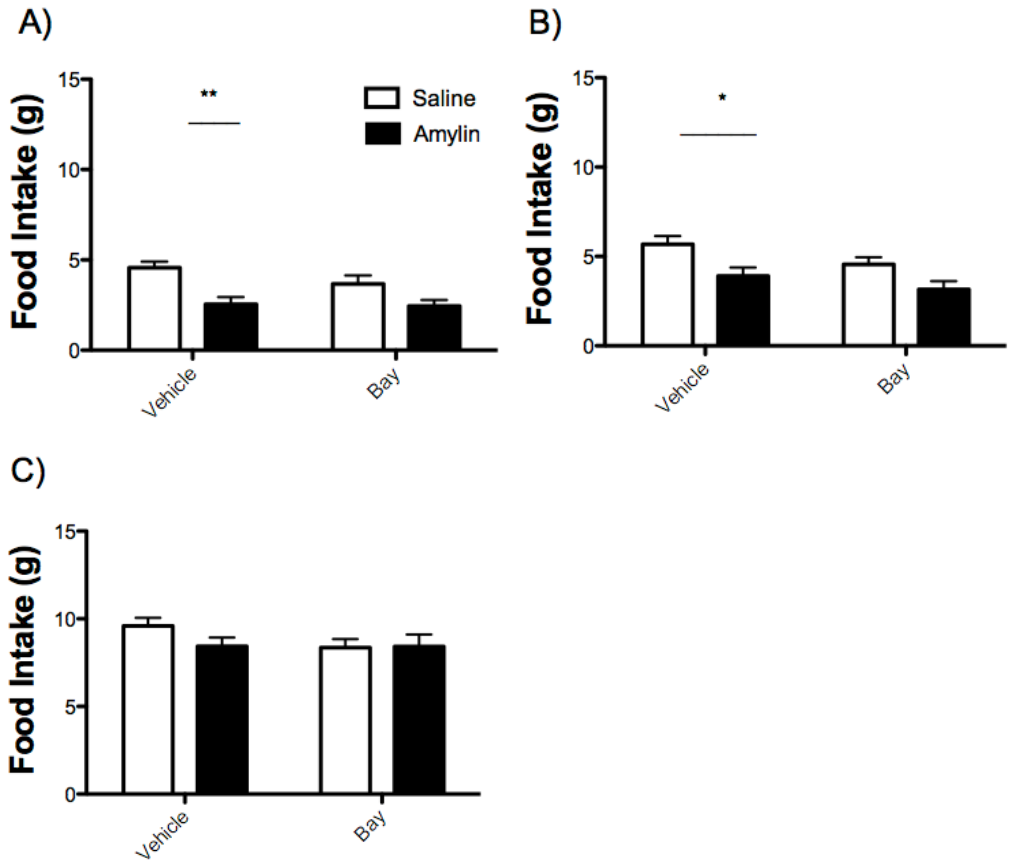

Figure 5.1: Mean ± SEM food intake 1 (A), 2 (B), and 4 h (C) after dark onset in rats pretreated with 4th ventricular vehicle or Bay 73-6691 (10 µg) 1.5 h before dark onset, followed by an injection of saline (white bars) or amylin (5 µg/kg i.p., black bars) immediately before dark onset. Symbols denote significant individual differences compared to the respective control group; **p<0.01, *p<0.05.

A significant reduction of food intake occurred in the animals which received vehicle and amylin treatment, in comparison to those treated with vehicle and saline. Food intake was decreased in the first hour by approximately 40%. Surprisingly, in the group which was pretreated with Bay 73-6691, amylin did not change food intake compared to saline control animals, and both groups seemed to have a tendency to eat less than the vehicle-saline control group (Fig. 5.1A). The reduction of food intake in the animals treated with vehicle-amylin was still visible after 2 hours (Fig. 5.1B), while 4 hours post-injection no significant effect was observed (Fig. 5.1C).

5.1.2 Results of Experiment 1b

Bolus central infusion of Bay 73-6691 with 20 µg/3 µl

As we did not observe an increased anorectic effect of amylin after Bay 73-6691 treatment in the first experiment, the dose of Bay 73-6691 was increased to 20 µg per infusion for the second experiment. Figure 5.2A demonstrates that in the first hour, there was an approximate 50% decrease of food intake in animals which received the vehicle-amylin treatment in comparison to the group receiving vehicle-saline ($p=0.01$). In the group receiving Bay 73-6691 pretreatment, we observed a tendency for amylin to decrease food intake, though this effect was not significant. In the second hour (Fig. 5.2B), there was a tendency for amylin to reduce food intake in both vehicle- and Bay 73-6691-treated groups. Four hours following dark onset (Fig. 5.2C), peripheral amylin significantly reduced food intake ($p<0.01$) in rats pretreated with Bay 73-6691. A similar trend was also present in the vehicle group, however the effect was not significant.

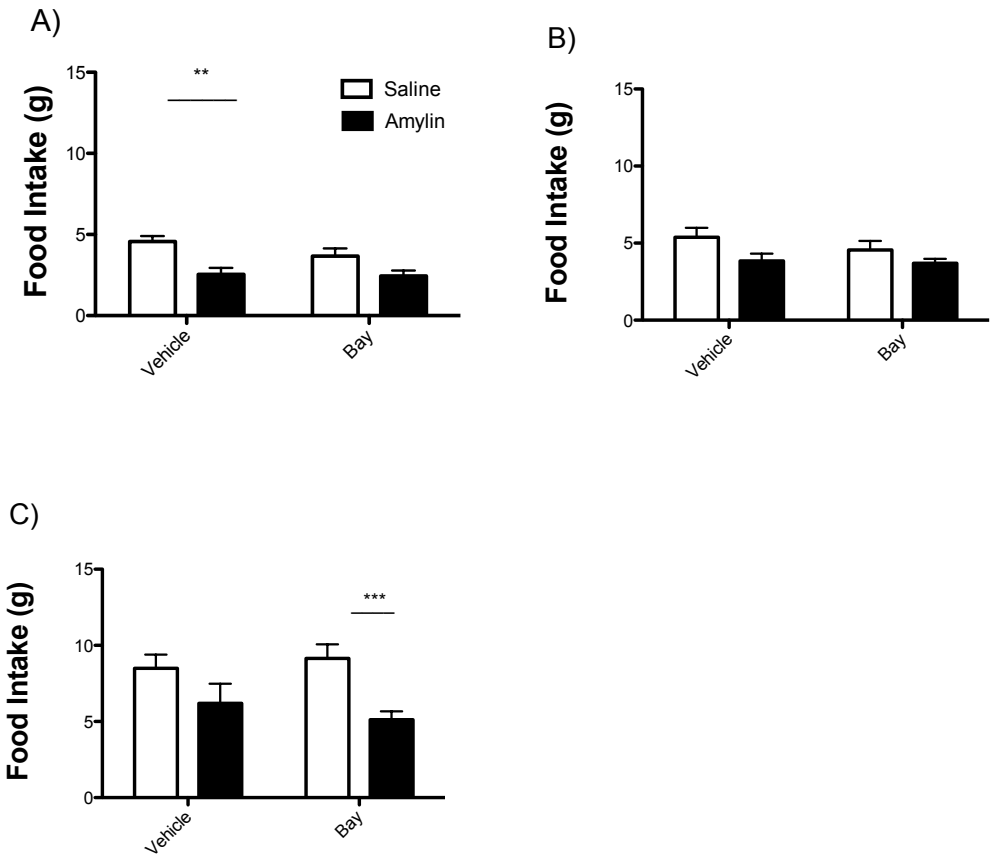

Figure 5.2: Mean ± SEM food intake 1 (A), 2 (B), and 4 h (C) after dark onset of rats pretreated with 4th ventricular vehicle or Bay 73-6691 (20 µg) 1.5 h before dark onset, followed by an injection of saline (white bars) or amylin (5 µg/kg i.p., black bars) immediately before dark onset. Symbols denote significant individual differences; **p<0.01, ***p<0.001.

5.1.3 Results of Experiment 1c

Bolus central infusion of Bay 73-6691 with a longer (3-hour) delay between Bay 73-6691 and amylin administration

Results from Experiment 1b suggested that Bay 73-6691 enhanced amylin action relatively late, i.e. only four hours after amylin administration, when a prolongation of amylin's anorexic effect was observed. Based on these results, in the third experiment, food was

removed 4 h prior to dark onset and Bay 73-6691 was infused into the 4th ventricle 3 h before dark onset at a dose of 20 μg/3 μl. As before, saline or amylin was given intraperitoneally 15 min before dark onset. As shown in Figure 5.3A, in the groups pretreated with vehicle, amylin treatment produced a significant decrease in food intake after one hour ($p<0.05$). Two and four hours after dark onset (Figs. 5.3B and 5.3C), amylin still had a tendency to reduce food intake in vehicle-pretreated groups, but this effect was no longer significant. There was no effect of amylin in rats pretreated with Bay 73-6691 at any time.

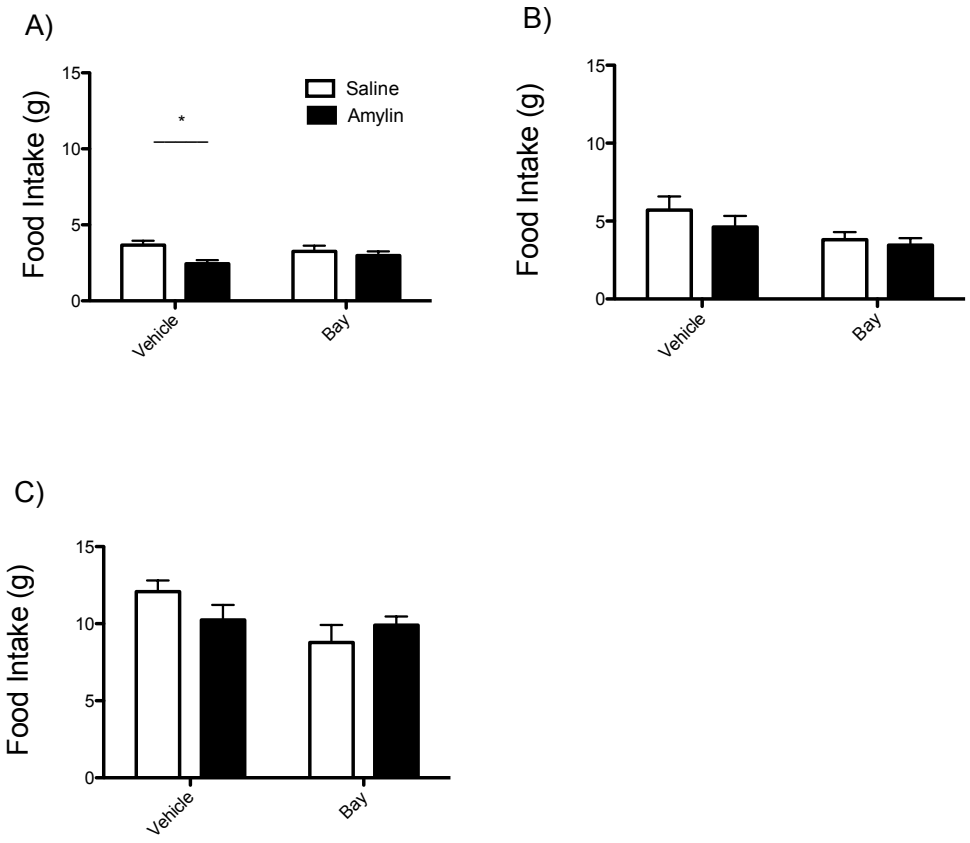

Figure 5.3: Mean ± SEM food intake 1 (A), 2 (B), and 4 h (C) after dark onset of rats pretreated with 4th ventricular vehicle or Bay 73-6691 (20 μg) 3.0 h before dark onset, followed by an injection of saline (white bars) or amylin (5 μg/kg i.p., black bars) immediately before dark onset. Symbols denote significant individual differences; *p<0.05.

5.1.4 Results of Experiment 1d

Effect of chronic central infusion of Bay 73-6691 on amylin action

Animals underwent a 4th ventricle cannulation surgery and were then implanted with minipumps. The minipumps were either filled with vehicle or Bay 73-6691, and released Bay 73-6691 at a dose of 6.66 µg/h (0.5 µl/h) for 14 days. Following implantation, rats were housed in BioDAQ® cages, which allowed us to continuously measure food intake. On test days (day 2 and 4), rats were fasted 2.5 hours before dark onset, and 15 min before dark onset, rats received saline or amylin (5 µg/kg s.c.).Food access was then given at dark onset. During the first two hours after dark onset (Figs. 5.4A and 5.4B), we observed that amylin had a clear tendency to reduce food intake in both groups of animals.

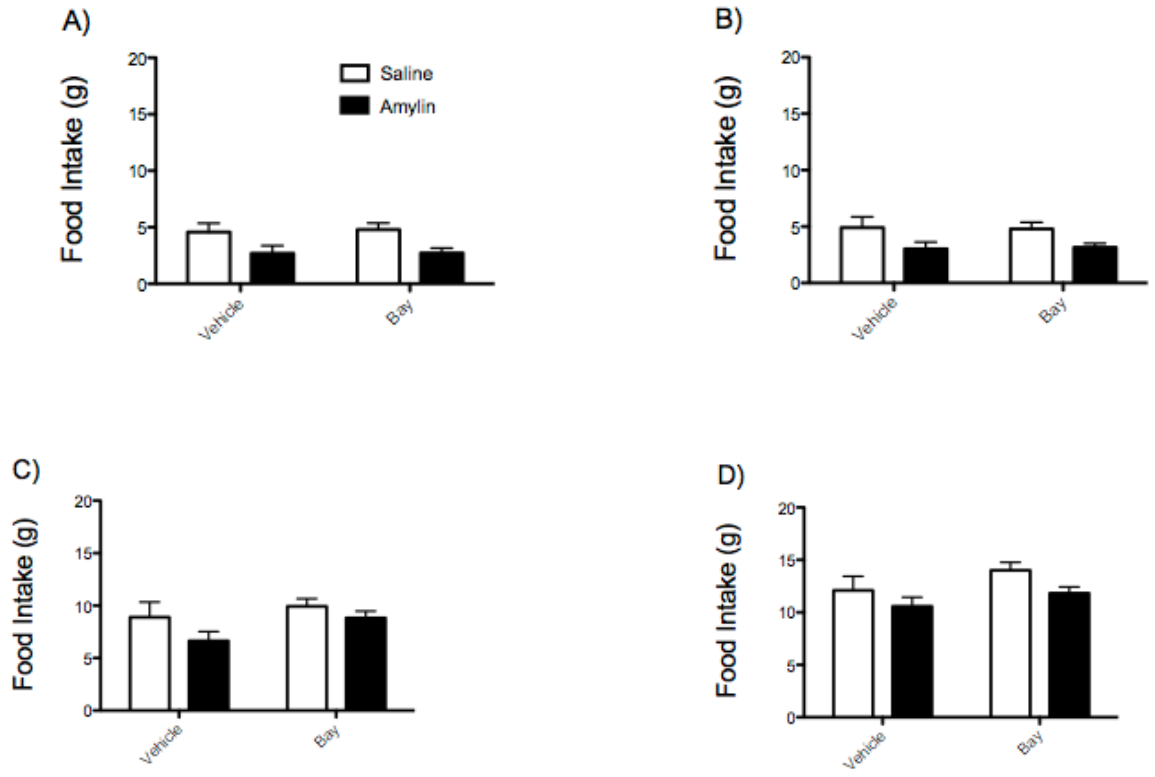

Figure 5.4: Mean ± SEM food intake 1 (A), 2 (B), 4 (C) and 5 h(D) after dark onset of rats with 4th ventricular chronic infusion of Bay 73-6691 (6.66 µg/h) or vehicle, followed by an injection of saline (white bars) or amylin (5 µg/kg i.p., black bars) immediately before dark onset.

There was no additional reduction of food intake in rats treated with the combination of chronic Bay 73-6691 and acute amylin compared to the vehicle-amylin group. Amylin's tendency to reduce food intake remained in the fourth hour (Fig. 5.4C) in the vehicle-treated group, but not in the Bay 73-6691-treated group. By the fifth hour after injection (Fig. 5.4D), there were no differences in food intake across groups

Analyses of meal patterns during chronic central infusion of BAY 73-6691

To assess if despite the lack of effect on cumulative food intake, chronic central infusion of Bay 73-6691 influenced baseline meal patterns, two non-test days were used to analyze feeding behavior, i.e. total 24h-food intake, average number of meals, average size of first and second meal, average latency to first meal, and average intermeal interval. Compared to vehicle-infused rats, chronic central infusion of Bay 73-6691 had no effect on the baseline number of daily meals (Fig. 5.5A), total daily food intake (Fig. 5.5B), size of first meal (Fig. 5.5C), size of second meal (Fig. 5.5D), nor the average length of the intermeal interval (Fig. 5.5F). Figure 5.5E demonstrates a slight tendency for Bay 73-6691 to decrease the latency to the first meal, compared to rats with chronic infusion of vehicle, but this effect was not significant.

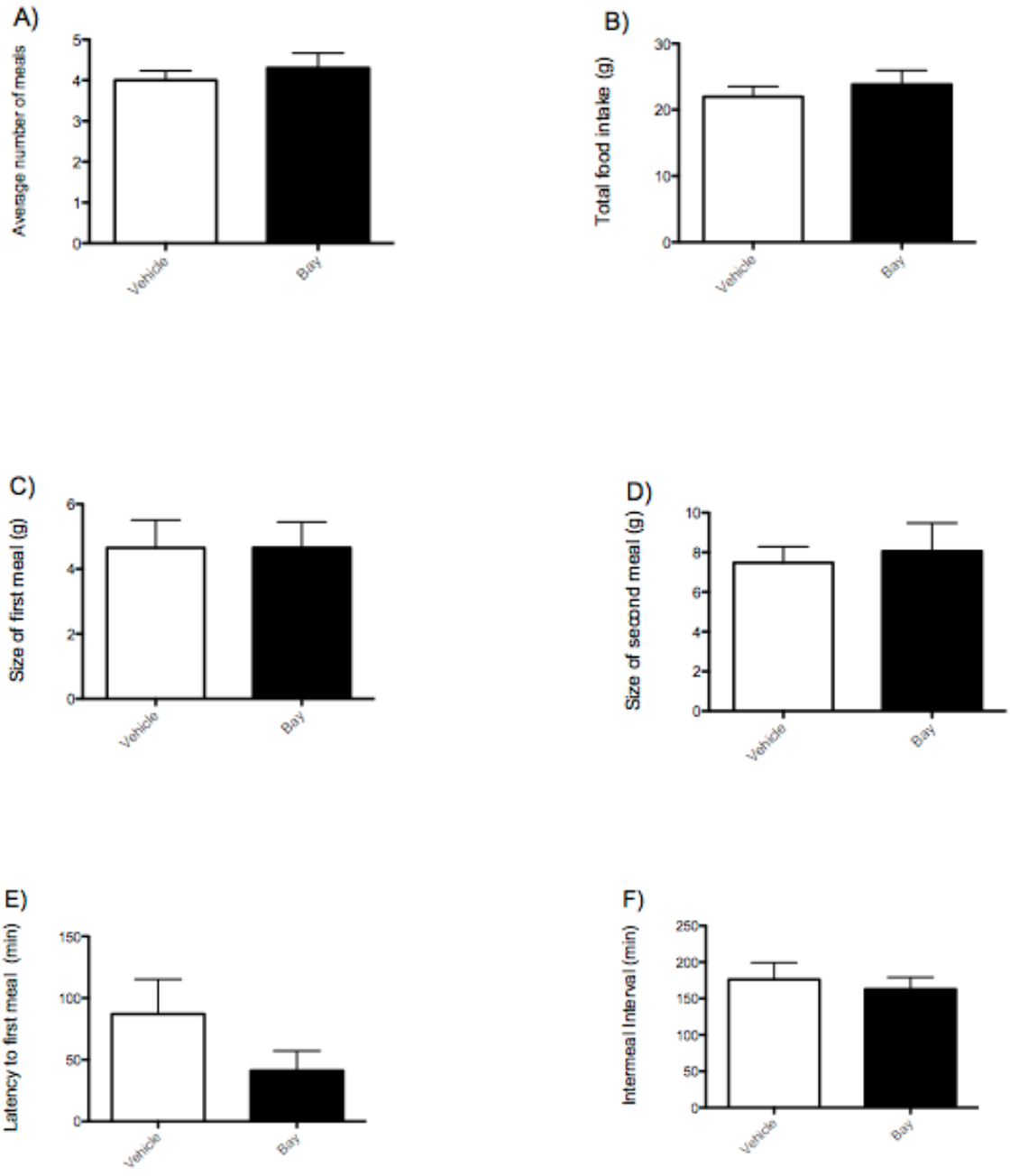

Figure 5.5: Mean ± SEM number of meals (A), total daily food intake (B), size of first meal (C), size of second meal (D), latency to the first meal (E), and intermeal interval (F) on a non-test day of rats with 4th ventricular chronic infusion of vehicle (white bars) or Bay 73-6691 (6.66 µg/h, black bars).

5.1.5 Results of Experiment 1e

Activation of c-Fos following chronic central infusion of Bay 73-6691

To assess if chronic central treatment with Bay 73-6691 enhanced the amylin-induced c-Fos formation, rats fitted with minipumps infusing vehicle or Bay 73-6691 were food deprived for 2.5 hours and shortly before dark onset received either vehicle or amylin (5 µg/kg; s.c.). Food was not returned, and 90 min later rats were perfused according to the c-Fos perfusion protocol described above. The brains were processed for free floating immunohistochemistry and stained against c-Fos. Figure 5.6 depicts the quantification of c-Fos positive cells in the area postrema. Amylin induced a slight, though not significant, increase in the number of c-Fos positive cells in rats chronically infused with vehicle. In rats treated chronically with central Bay 73-6691, there was a significant increase of c-Fos in the area postrema. Representative images of c-Fos labeling in the area postrema are shown in Figure 5.7.

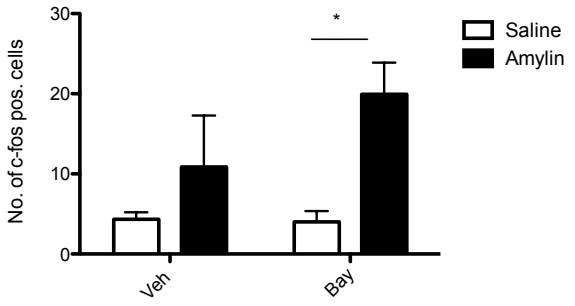

Figure 5.6: Male Sprague Dawley rats underwent 4th ventricle cannulation surgery and minipump implantation, loaded with Bay 73-6691 (6.66 µg/h) or vehicle, followed by an injection of saline (white bars) or amylin (5 µg/kg s.c., black bars) just before dark onset. Animals were perfused 1.5 h afterwards. C-Fos positive cells in the AP were counted. Symbols denote significant individual differences; *p<0.05.

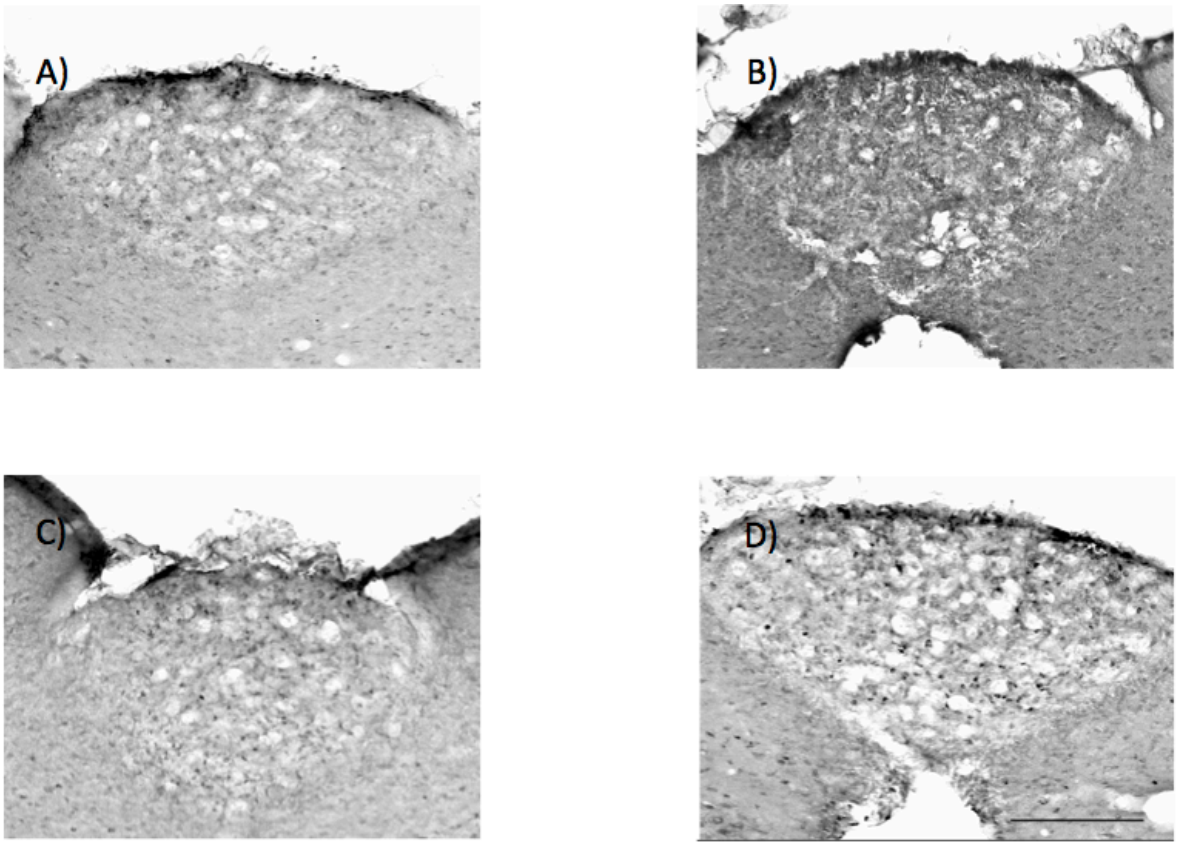

Figure 5.7: Representative brightfield photomicrographs showing c-Fos immunoreactivity in the area postrema 90 min after treatment with vehicle-saline (A), Bay 73-6691-saline (B), vehicle-amylin (C) and Bay 73-6691-amylin (D). Scale bar = 200 *µ*m

5.2 Effect of peripheral administration of Bay 73-6691 on amylin action

5.2.1 Results of Experiment 2a

Peripheral administration of Bay 73-6691

To assess if peripheral administration of Bay 73-6691 enhances amylin's anorectic action, rats were pretreated peripherally with either vehicle or Bay 73-6691 (0.1 mg/kg; i.p.) 1.5 hours before dark onset, followed by treatment with saline or amylin (5 µg/kg; i.p.) shortly before

dark onset. Figure 5.8A shows that there was no difference in food intake between vehicle-treated rats which received saline or amylin. In the group which received Bay 73-6691, amylin had a tendency to reduce food intake, though this effect was not significant. Similar results were observed two hours (Fig. 5.8B), and four hours after dark onset (Fig. 5.8C); i.e., there were no differences in food intake across all groups.

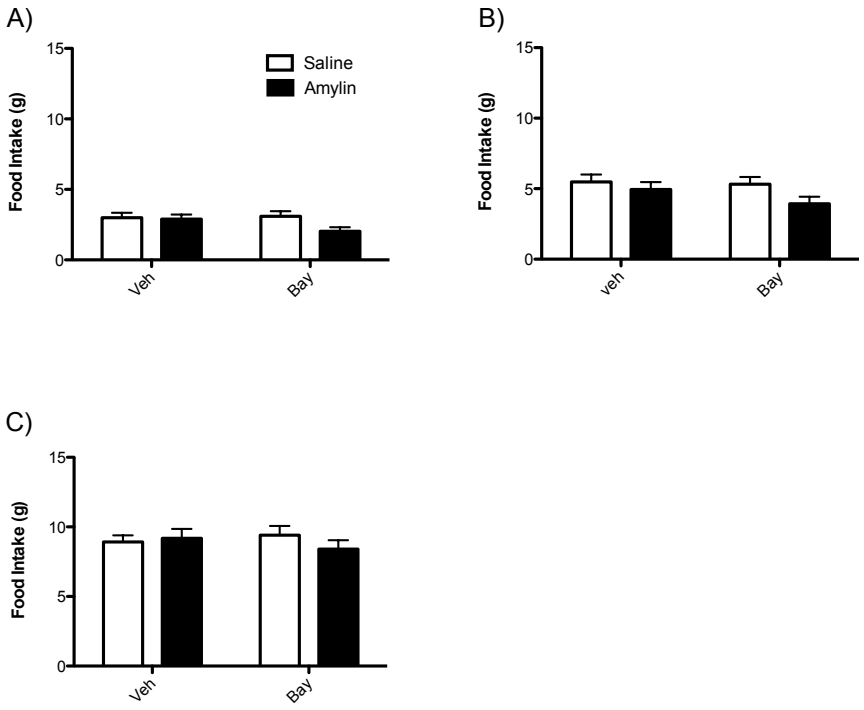

Figure 5.8: Mean ± SEM food intake 1 (A), 2 (B), and 4 h (C) after dark onset of rats pretreated vehicle or Bay 73-6691 (i.p.; 0.1 mg/kg) 1.5 h before dark onset, followed by an injection of saline (white bars) or amylin (5 µg/kg;black bars) immediately before dark onset.

5.2.2 Results of Experiment 2b

Conditioned Taste Aversion (CTA)

Under some conditions, Bay 73-6691 appeared to reduce food intake compared to vehicle-saline treated rats, i.e. even in the absence of amylin. We therefore tested if peripheral administration of Bay 73-6691 itself may affect eating, possibly by the induction of a condition taste aversion. Rats were trained under the CTA paradigm described previously (le Roux et al., 2011); on the final test day, rats were offered a choice of water or saccharine; this allowed us to determine if Bay 73-6691 induced feelings of malaise in rats. Figure 5.9 shows that on the final test day, regardless of treatment with vehicle or Bay 73-6691, all rats showed a significant preference to drink saccharine versus water during a 30 min test, indicating that there was no evidence for BAY 73-6691 to induce a CTA.

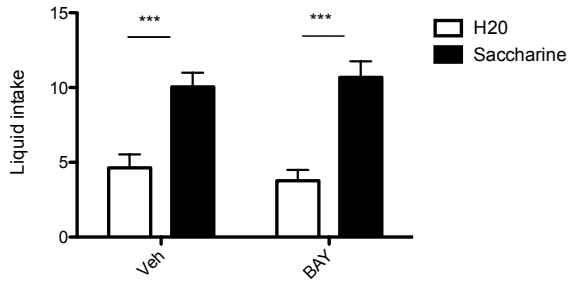

Figure 5.9: Mean ± SEM intake of water (white bars) and saccharine (black bars) during the final 30 min test of the CTA paradigm in rats treated with vehicle or Bay 73-6691. Symbols denote significant differences between water and saccharine intake; ***p<0.001.

5.2.3 Results of Experiment 2c

Sensitivity to amylin as assessed by c-Fos activation

A critical component of every experiment in this part of the dissertation was a reliable and robust anorectic effect of amylin. Unfortunately and unexpectedly, this effect was rarely observed. To assess the rats' sensitivity to amylin, various doses of amylin were injected

peripherally, and the accumulation of c-Fos, a marker of neuronal activation, was semi-quantified in the area postrema to determine if the rats responded to amylin. CCK-induced c-Fos was used as a positive control. Rats were fasted 2.5 hours before dark onset and shortly before dark onset injected with either saline, amylin (5 μg/kg, 20 μg/kg, or 50 μg/kg) or CCK (3 μg/kg). Rats were perfused 90 min later and the hindbrains were processed for c-Fos immunohistochemistry. The number of c-Fos positive cells was estimated in a semiquantitative way, using the following evaluation of c-Fos cells:

- = <10 positive cells

+ = 10 positive cells

++ = 20 positive cells

+++ = 30 positive cells

Treatment	c-Fos signaling
Saline	-
Amylin 5 μg/kg	+
Amylin 20 μg/kg	-
Amylin 50 μg/kg	+++
CCK 3 μg/kg	+++

Table 5.1.1: Semi-quantification of c-Fos positive cells in area postrema slices.

Figure 5.10A shows a representative rat that received saline, which resulted in c-Fos activation in just a few cells. Figures 5.10B, C, and D depict representative brain sections of rats treated with increasing doses of amylin. Only animals treated with the highest dose of amylin (50 μg/kg) showed a clear c-Fos response in the AP. A CCK-treated rat is depicted in Figure 5.10E; this comparably low dose resulted in the most robust c-Fos response of all treatment groups. Hence, for reasons that are unknown to us, the rat strain used in these experiments seemed to be relatively insensitive to the actions of amylin when compared to previous studies where amylin appeared to be more potent than CCK when given at comparable doses (Lutz et al., 1998; Reidelberger et al., 2001; Young et al., 1996).

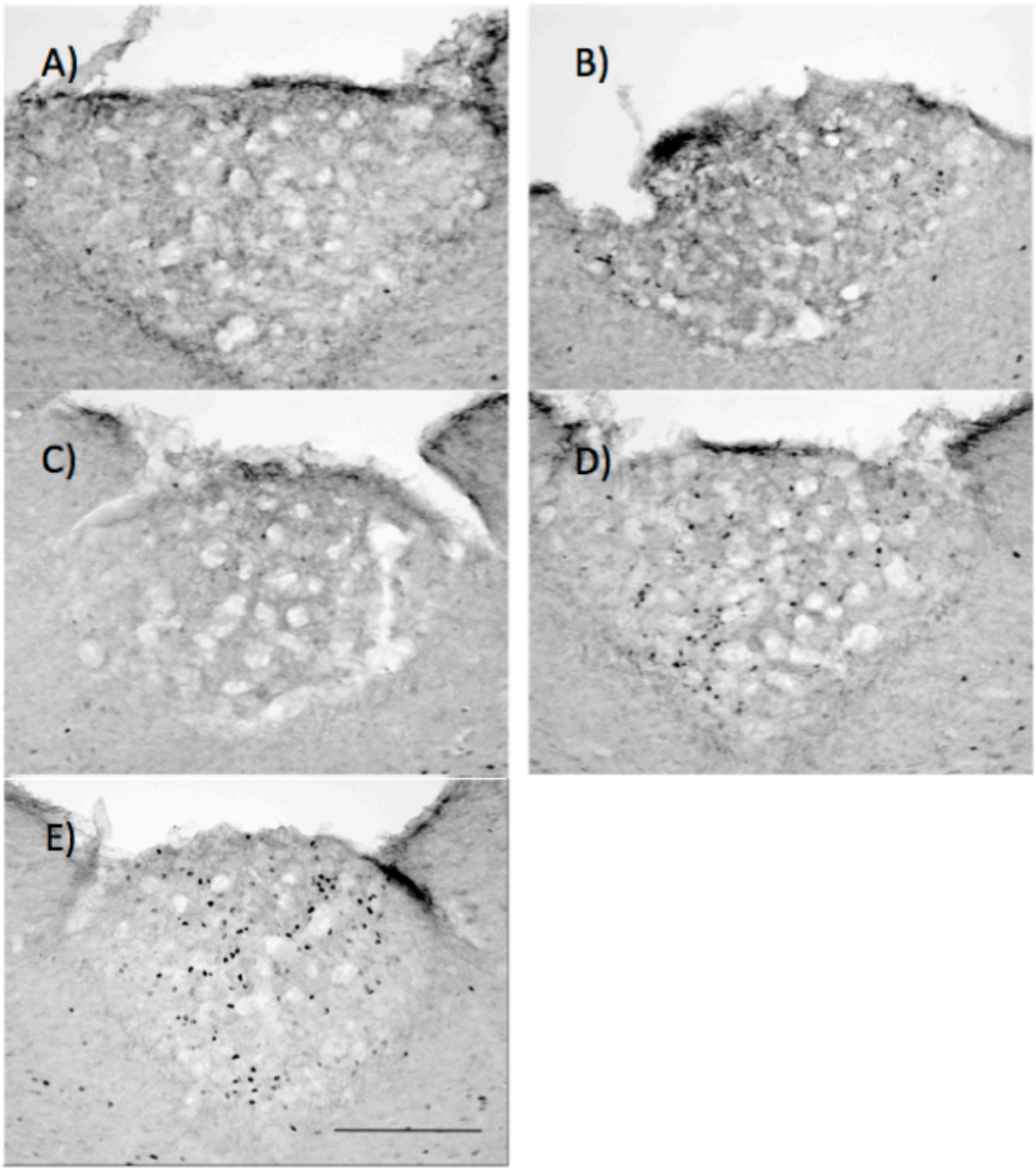

Figure 5.10: Representative brightfield photomicrographs showing c-Fos immunoreactivity in the area postrema 90 min after treatment with saline (A), 5 µg/kg amylin (B), 20 µg/kg amylin (C), 50 µg/kg amylin (D), or 3 µg/kg CCK (E). Scale bar = 200 μm

5.3 The role of the AP in amylin-induced restoration of leptin sensitivity

5.3.1 Identification of DIO and DR rats after HFD

Rats were maintained on a HFD for 8 weeks before the area postrema lesions were performed. After two weeks on HFD, rats were designated diet-induced-obese (DIO) or diet-resistant (DR) according to their body weight gain (Fig. 5.11). For the purpose of this study, rats gaining over 30% of the initial BW in these two weeks were considered DIO, while those gaining less than 30% were designated DR.

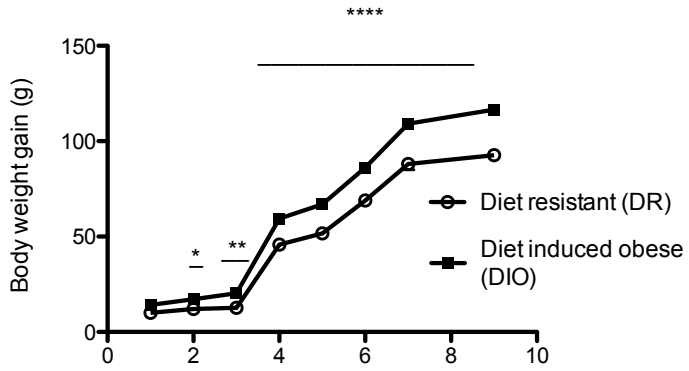

Figure 5.11: Body weight gain of the DR (empty circles) and DIO rats (black squares) maintained on HFD from week 1. Symbols denote significant individual differences between groups; *p<0.05; **p<0.01; ***p<0.001.

5.3.2 Results of Experiment 3a

Area postrema lesion and area postrema lesion verification test

DIO rats underwent area postrema lesion (APX) surgery 8 weeks after exposure to the HFD. To determine if the lesions were successful and complete, a verification test was performed, where the food intake response following amylin treatment (5 μg/kg, i.p.) was recorded. The preset criterion was that lesioned rats showing 15% or greater reduction of food intake following amylin treatment would not be included in the study.

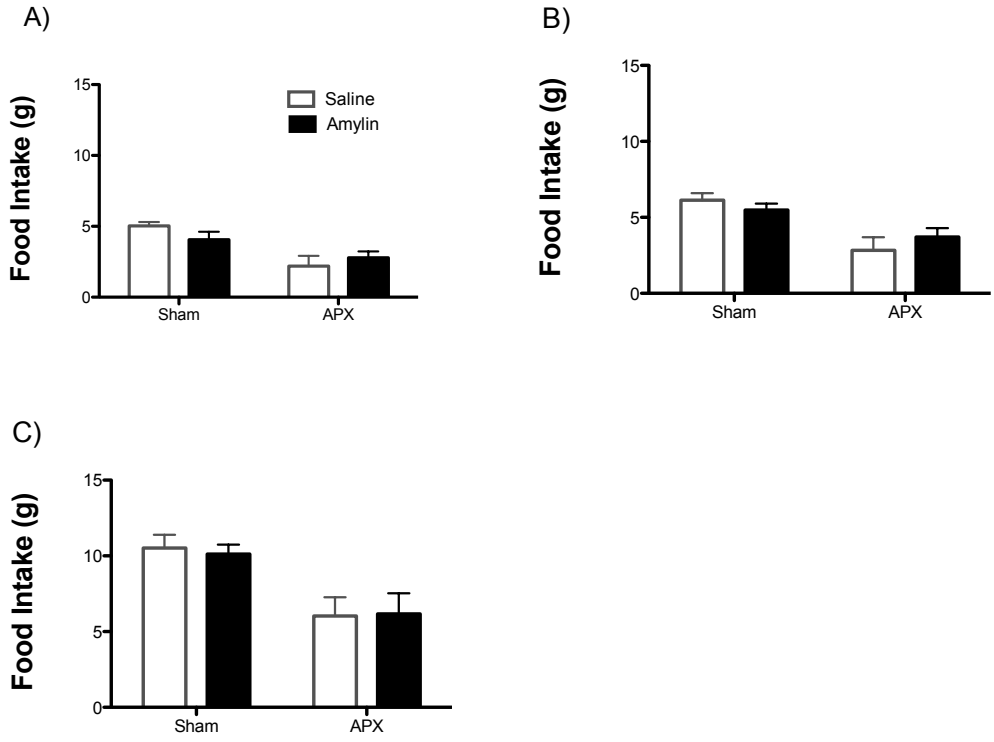

Figure 5.12.: Mean ± SEM food intake 1 (A), 2 (B), and 4 h (C) after dark onset of sham- and area postrema-lesioned rats treated with saline (white bars) or amylin (5 µg/kg i.p., black bars) immediately before dark onset.

At no time point following the injection did amylin reduce food intake in APX rats (Figs. 5.12A-C). Though there was tendency for amylin to reduce food intake in sham-lesioned rats 60 min after application (Fig. 5.12A), this effect unfortunately was not significant and a clear pre-mortem distinction between operation groups was therefore not possible.

5.3.3 Results of Experiment 3b

Effect of chronic peripheral infusion of amylin on body weight

Sham- and AP-lesioned DIO rats were then surgically implanted with minipumps that infused saline or amylin (100 µg/kg/day; s.c.) for 14 days. Figure 5.13A shows that sham-lesioned

animals infused with amylin showed a significant reduction in body weight gain compared to shams receiving saline ($p<0.05$). Lesioning the AP blocked this effect, so that no difference in body weight gain was observed in APX rats infused with saline or amylin (Fig. 5.13B). Figure 5.13C depicts the changes in body weight for all four groups over the entire duration of the study.

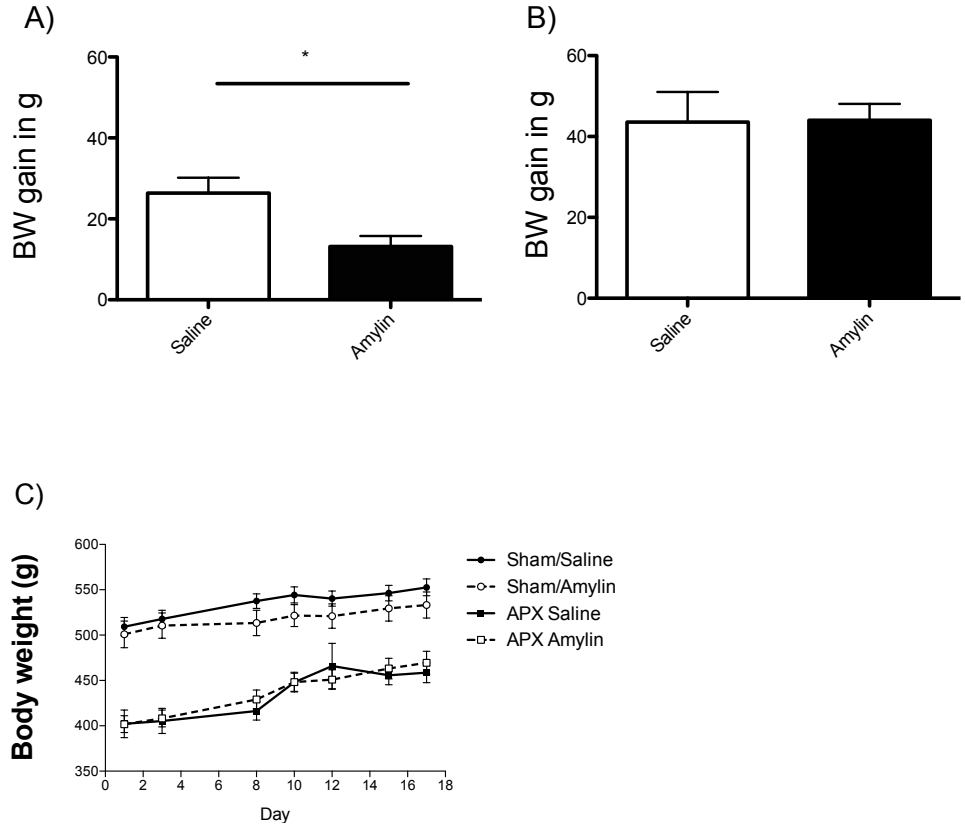

Figure 5.13: BW gain of sham rats after minipump implantation (A), BW gain of APX rats after minipump implantation (B), data are from day 17 after implantation of minipump ;BW development over time of all groups after minipump implantation (C). Rats carrying saline minipumps are shown in white bars, and those with amylin minipumps are shown in the black bars. Symbols denote significant differences between saline and amylin treatment; *p<0.05

While amylin reduced the rate of body weight gain in sham-lesioned rats, APX rats showed no difference in body weight development regardless of the minipump infusate. Interestingly, APX rats generally gained more weight than sham-lesioned rats during the 2-week experimental period.

5.3.4 Results of Experiment 3c

Effect of amylin infusion on leptin-induced anorexia

Chronic infusion of amylin reportedly restored leptin sensitivity in obese leptin resistant rats (Roth et al., 2008). Here we tested if amylin had the same effect under our experimental conditions and whether amylin's leptin-sensitizing effect was also seen in DIO rats after lesioning the AP. We first assessed leptin-induced anorexia following treatment with amylin for one week. Sham and APX rats equipped with minipumps infusing saline or amylin were fasted for the last 2 hours of the light phase, and shortly before dark onset received an injection of either distilled water or leptin (5 mg/kg, i.p.). Food intake was measured 4 h (Fig. 5.14A) and 24 h (Fig. 5.14B) after injection. Surprisingly, and in contrast to our expectations based on the study by Roth and colleagues (Roth et al., 2008), leptin did not reduce food intake under any of the conditions tested.

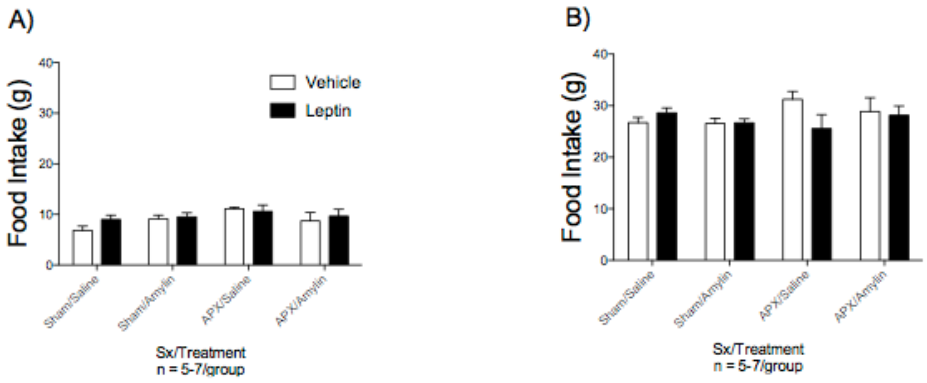

Figure 5.14.: Mean ± SEM food intake 4 (A), and 24 h (B), after dark onset of sham or APX rats pretreated with subcutaneous, chronically-infused vehicle or amylin (100 µg/kg/day), followed by an injection of saline (white bars) or leptin (5 mg/kg i.p., black bars) immediately before dark onset.

5.3.5 Results of Experiment 3d

Effect of amylin infusion on leptin-induced STAT3 phosphorylation

It was also reported that amylin's effect to restore leptin sensitivity coincides with an enhanced leptin-induced STAT3 phosphorylation in the VMH of DIO rats. Thus, in a follow-up experiment, we tested if amylin treatment restores leptin-induced phosphorylation of STAT3 in DIO rats after lesioning the AP. As before, rats were fasted for 2 hours, treated with distilled water or leptin (5 mg/kg, i.p.) before dark onset, and perfused 45 min later according to the pSTAT3 protocol described above.

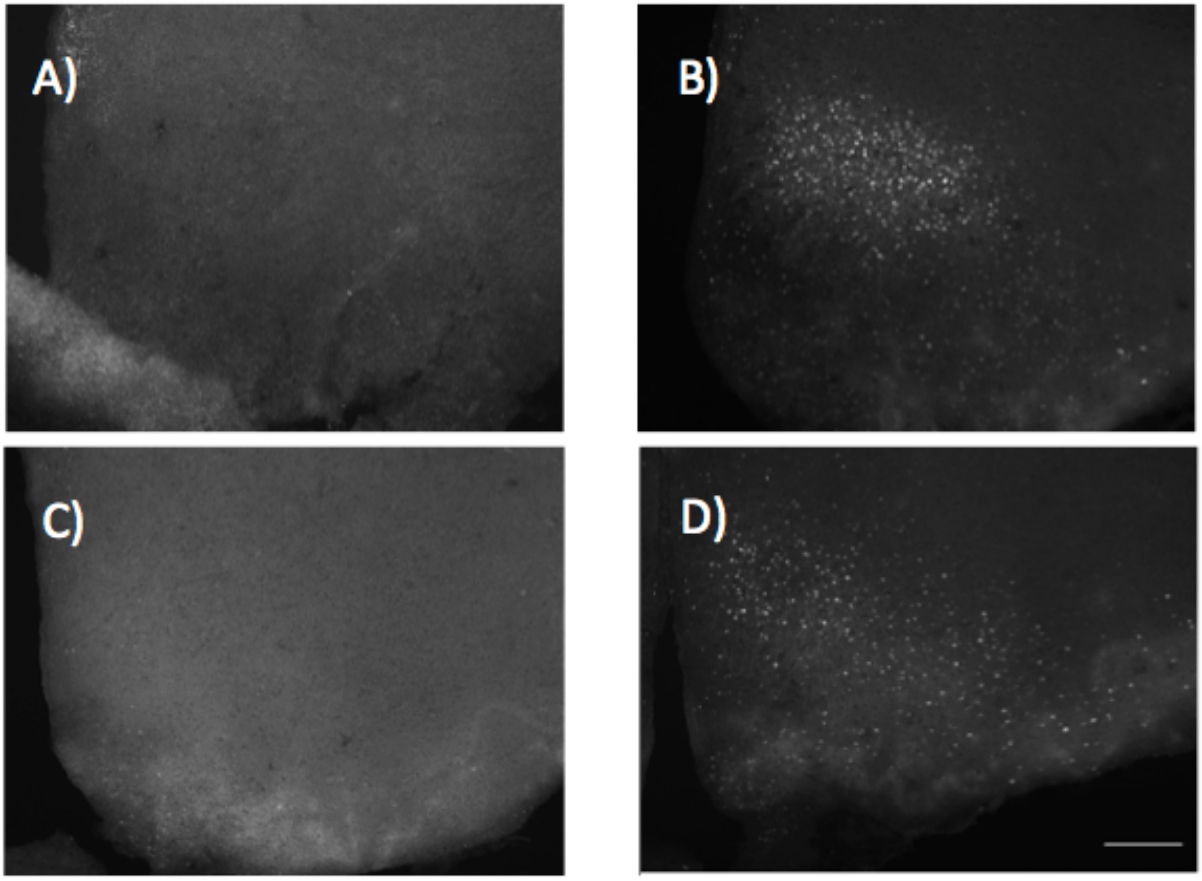

Figure 5.15: Representative fluorescent photomicrographs showing pSTAT3 immunoreactivity in the VMH 45 min after treatment with vehicle (A) or leptin (B) in sham rats infused with NaCl for 14 days; or after treatment with vehicle (C) or leptin (D) in sham rats infused with amylin(100 µg/kg/day) for 14 days. Scale bar = 200 *µ*m

Hypothalamic brain slices were processed for pSTAT3 immunohistochemistry, photomicrographs of the sections were captured, and the pSTAT3-positive cells within the VMH were quantified. The leptin-induced pSTAT3 response in saline and amylin treated sham-lesioned rats is shown in Figure 5.15. Leptin induced a comparable number of pSTAT3 in the VMH of rats infused with either saline (Fig. 5.15B) or amylin (Fig. 5.15D) for 14 days .

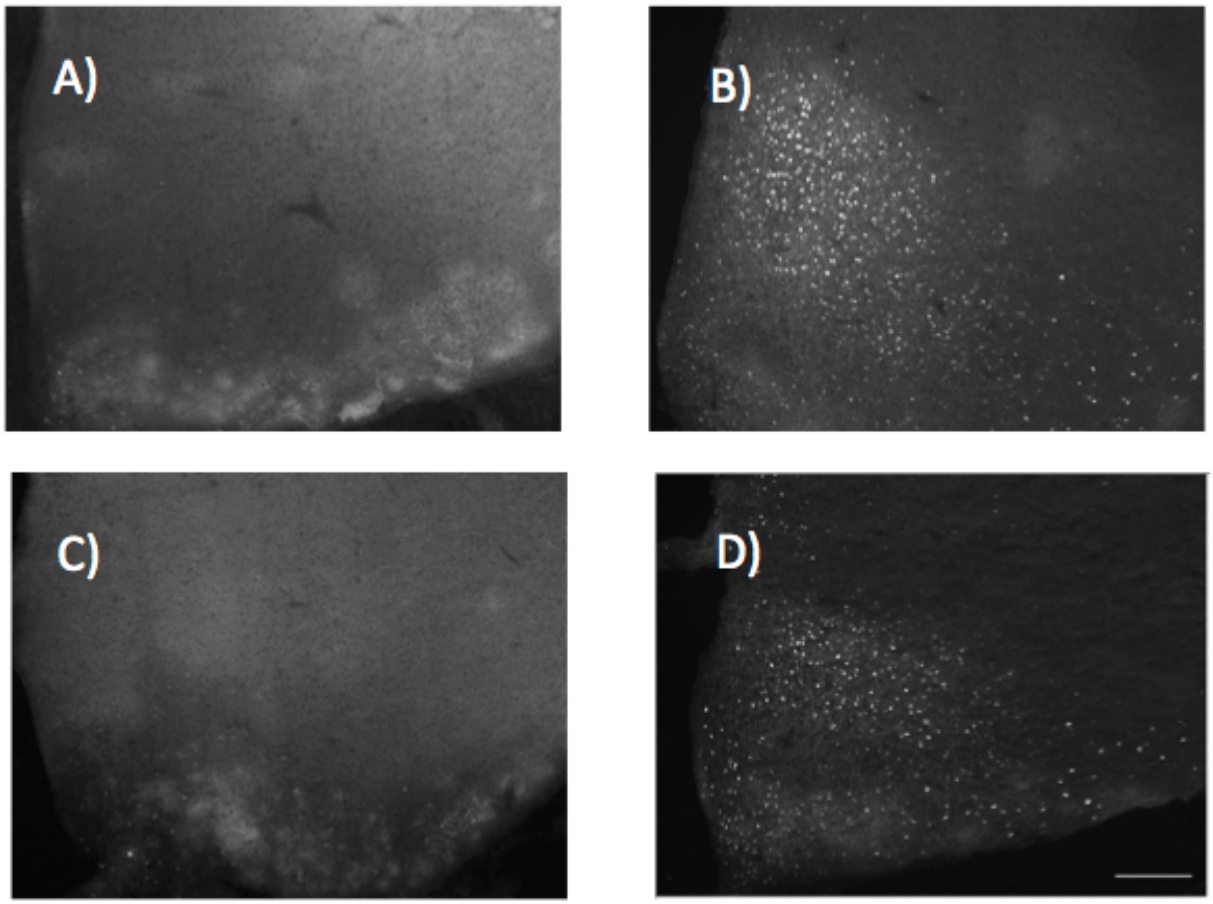

Figure 5.16: Representative fluorescent photomicrographs showing pSTAT3 immunoreactivity in the VMH 45 min after treatment with vehicle (A) or leptin (B) in APX rats infused with NaCl for 14 days; or after treatment with vehicle (C) or leptin (D) in APX rats infused with amylin (100 µg/kg/day) for 14 days. Scale bar = 200 *µ*m

Figure 5.16 shows photomicrographs of animals which underwent an area postrema lesion before testing. Compared to sham rats, leptin treatment resulted in a comparable number of pSTAT3-postive cells in the VMH of AP-lesioned rats infused with saline (Fig. 5.16B) or

amylin (Fig. 5.16D) for 14 days. The number of pSTAT3-postive cells in leptin-treated APX rats was also similar that in sham-lesioned rats, as can be seen in the quantification shown in Figure 5.17. Hence, an intact AP did not seem to be necessary for this effect of leptin.

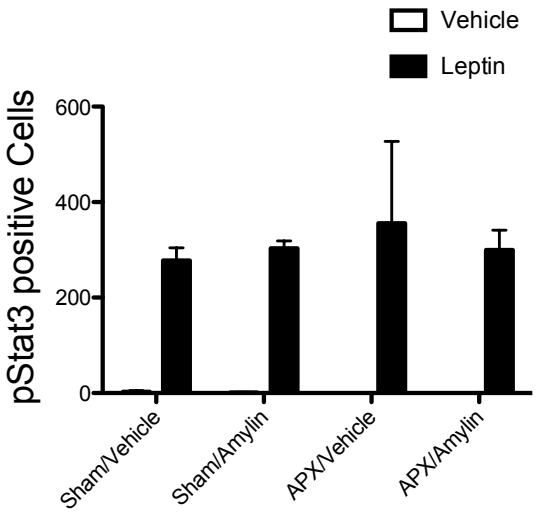

Figure 5.17: Quantification of pSTAT3 immunoreactivity in slices of VMH collected from sham and APX rats treated with saline vehicle or amylin (100 µg/kg/day) for 14 days, followed by acute injection of vehicle (white bars) or leptin (5 mg/kg i.p.; black bars) 45 min before sacrifice.

6 Discussion

6.1 Does Bay 73-6691 prolong amylin's anorectic action?

The aim of this study was to test the hypothesis that the PDE9 inhibitor, Bay 73-6691, may enhance and prolong amylin's anorectic action by blocking the degradation of cGMP, the second messenger mediating the effect of amylin. It is critical to initially state that throughout our experiments we encountered the problem that the ability of amylin to reduce food intake was often blunted or absent. Since amylin's anorectic effect was the basis for testing the effect of Bay 73-6691, it is difficult to evaluate the results of some trials. In the cases that we observed a reliable amylin effect, we could see four main findings:

1) Bay 73-6691 did not prolong the anorectic effect of amylin under most conditions. 2) The results suggest that the acute administration of Bay 73-6691 alone had a small anorectic effect, possibly by enhancing the effects of endogenous amylin or other satiating peptides. 3) These results did not seem to occur due to malaise, since Bay 73-6691 did not elicit a conditioned taste aversion. 4) Finally, we observed that rats pretreated with Bay 73-6691 showed a significantly higher number of amylin-induced c-Fos-positive cells in the area postrema, compared to animals that received the vehicle/amylin treatment.

6.1.1 Effect of central administration of Bay 73-6691 on the action of peripheral amylin

In the trials where Bay 73-6691 was administered centrally and acutely, we did not observe an enhancing effect of PDE9 inhibition on amylin's anorectic effect; however we saw a tendency for Bay 73-6691 on its own to reduce food intake. In experiment 1a, amylin significantly reduced food intake for the first 2 hours in rats pretreated with vehicle. The group which received Bay 73-669 pretreatment followed by vehicle consumed a similar amount of food as the vehicle/amylin treated group. It seemed that Bay 73-6691 was sufficient to slightly reduce eating, similar to the effect produced by amylin alone. This raised the question if Bay 73-6691 might enhance the action of endogenous amylin, or other satiating hormones. However, when Bay 73-6691 was given in combination with exogenous amylin, we did not observe a

heightened anorectic effect, but rather an effect similar to the one seen in the Bay 73-6691/vehicle group.

To determine if the dose of Bay 73-6691 was just too low to enhance exogenous amylin, we increased the dose of Bay 73-6691 to a 20 μg bolus injection in experiment 1b. Under these conditions, we observed a significant reduction in food intake after 4 hours in the Bay 73-6691/amylin group, as compared to the Bay 73-6691/vehicle group; at this time point, amylin alone had no longer an effect of eating. This suggests an interaction of amylin and the PDE9 inhibitor, indicating therefore that Bay 73-6691 prolonged amylin's action. As observed in experiment 1a, the Bay 73-6691/vehicle group again displayed a reduction in food intake, compared to the vehicle only group, during the first 2 hours similar to the vehicle/amylin group.

According to the outcome of experiment 1b we postulated that the timeframe of Bay 73-6691's action might be different than the previously-suggested half life of appoximately 2 hours (van der Staay et al., 2008). We therefore designed an additional experiment in which we took into account the potentially longer time to the onset of the drug's action. However, even with a longer delay of 3 hours between Bay 73-6691 and amylin treatments, neither an effect of amylin nor of the PDE9 inhibitor was seen. Aside from the data by van der Staay and colleagues, little is known about the time course of Bay 73-6691 action on cGMP levels. A recent study showed that subcutaneous administration of another PDE9 inhibitor, PF-4449613, elevated cGMP concentration in cerebrospinal fluid for four hours after treatment, with the peak effect observed between 30 and 60 minutes (Kleiman et al., 2012) Unfortunately, similar experiments have not been carried out examining Bay 73-6691's effect on central cGMP levels. Thus, it would be advantageous to develop techniques that would allow us to track changes in cGMP in the area postrema following BAY 73-6691 treatment, and then determine the period of highest activity of Bay 73-6691, as well as additional pharmacokinetic details.

Due to the highly vascularized area postrema, we questioned whether acute infusions of Bay 73-6691 into the 4th ventricle might be rapidly washed out prior to interacting with neurons of the AP. Thus, we designed an experiment where the animals received a chronic infusion of the PDE9 inhibitor into the 4th ventricle. Variants of the acute central trials described before

were repeated, but Bay 73-6691 was administered chronically into the 4th ventricle by using minipumps, and animals were housed in BioDAQ® cages to continuously monitor feeding behavior. Unfortunately and for unknown reasons, rats did not show a significant anorectic response to amylin under these test conditions. Further, chronic central treatment with Bay 73-6691 had no additional effect on feeding behavior, regardless of acute treatment with saline or amylin. Because little is known about the chemical properties of Bay 73-6691 under our test conditions, we cannot rule out that Bay 73-6691 did not remain stable and active in DMSO for 14 days. The experiment was also difficult to evaluate because we did not have a secondary marker to confirm that Bay 73-6691 actually penetrated the brain and modulated cGMP levels in the predicted way.

We took advantage of the continuous food intake monitoring by the BioDAQ® system to determine if there were any differences in baseline meal parameter between vehicle- and PDE9 inhibitor-treated rats. Analysis of these data revealed no difference in meal size, meal number, IMI, latency to feed, or total food intake between the rats receiving chronic central treatment of vehicle or Bay 73-6691. While we did not observe an effect of Bay 73-6691, the results of the meal pattern analysis was somewhat surprising. Rats of similar size and body weight had been reported to consume an average of 9.4 nocturnal meals averaging in size of 2.3 g with an average intermeal interval of 65.5 min (Zorrilla et al., 2005). Compared to these baseline meal properties, both groups of animals in our study consumed fewer (4) but larger (6-7 g) meals, and the latency to the first meal in the dark phase was delayed by about 45 to 90 min after dark onset. In fact, the meal patterns of both groups bear some resemblance to those observed in rats following a lesion of the AP (Stricker et al., 1997; Brägger, unpublished findings). This posed the question of whether these changes in feeding behavior originated from chronic central infusion of a DMSO-based vehicle. While the potential side effects of DMSO application are discussed in greater detail in the following section, these results suggest that DMSO may have caused some damage to the area postrema neurons leading to an alteration in meal patterns and subsequently to the sensitivity to amylin. On the other hand, the results of the terminal c-Fos study in these chronically-infused animals suggested that this may not have been the case, because a c-Fos response in at least some AP neurons was still detectable. Furthermore, the latter results showed that even if treatment with Bay 73-6691 did not have an impact on food intake, it did enhance amylin-induced c-Fos

signaling in the AP; rats receiving Bay 73-6691 and amylin had a significantly higher number of c-Fos-positive cells than rats treated with Bay 73-6691 and saline, which was an effect not observed in rats chronically infused with vehicle. Therefore, if DMSO had a negative effect on the neurons in the area postrema, it certainly did not cause damage to all of them; nonetheless, it cannot be excluded that such a high concentration of DMSO (75% of total volume) might partially damage neurons in the AP.

6.1.2 Effect of peripheral administration of Bay 73-6691 on amylin action

Corresponding to what we had observed after central administration of Bay 73-6691, neither an effect of amylin nor an effect of the PDE9 inhibitor was detectable in studies where Bay 73-6691 was administered peripherally. Because we failed to produce a reliable anorectic effect of amylin in the first trial, we decided to repeat this experiment with a different strain of rats and by modifying the route of amylin administration; however, these attempts also failed to produce an effect of amylin on food intake. The lack of an amylin effect under vehicle control conditions made it difficult to analyze the data and to interpret a potential role of Bay 73-6691 in modulating amylin action. Because we observed no effect of amylin on feeding behavior, we suspected that the rats were insensitive to amylin and designed an experiment to verify our suspicion. Hence, in a terminal experiment, animals were peripherally treated with different concentrations of amylin or with a control treatment of CCK, and the brains were then processed for c-Fos immunohistochemistry. The results clearly showed that even 50 µg/kg amylin induced a weaker c-Fos expression in the AP than the the control injection of 3 µg/kg CCK; lower doses of amylin induced very little or no c-Fos in the AP. These results suggest that these rats exhibited blunted sensitivity to amylin. The diminished sensitivity was evident throughout our set of experiments, because amylin often failed to induce a sufficient and reliable anorectic effect. Because we did not observe increased expression of c-Fos in the AP of amylin-treated rats, we first assumed that the quality or the level of sensitivity of the c-Fos antibody was deficient. However, this theory was contradicted by the satisfactory CCK-induced c-Fos response. The relatively short fasting in this experiment time could have been another critical issue. We used a fasting time of 2.5 h, and while it is possible that a longer fasting time would have led to a stronger c-Fos response (Michel et al., 2007; Zuger et al., 2013), we observed in experiment 1f that 2.5 h was actually sufficient to induce a clear c-Fos

response. Further, in experiment 1d, we included control brain tissue from a rat which was also fasted for exactly 2.5 h, and received an injection 5 µg/kg amylin at dark onset, the only difference was that no vehicle or Bay 73-6691 was administered. This control rat displayed a clear c-Fos response, compared to brain slices from all other rats, suggesting that the fasting time was less of an issue, but that the infusion of vehicle or Bay 73-6691 could have somehow contributed to the blunted c-Fos response.

It is unclear why in most experiments of this study amylin did not induce an anorectic effect. We hypothesized that there might be a connection to the dimethyl sulfoxide (DMSO) that was used as a vehicle for Bay 73-6691. DMSO is an amphipathic molecule, with a highly polar domain and two apolar groups, which makes it a very good solvent for different drugs; however, DMSO is not an inert substance. DMSO itself is used as a treatment for different diseases like the amelioration of interstitial cystitis (Parkin et al., 1997), rheumatologic diseases, dermatologic diseases and chronic prostatitis (Shirley et al., 1978). Treatment of traumatic brain edema (Ikeda and Long, 1990) relies on DMSO's ability to cross the blood brain barrier (Broadwell et al., 1982), and it also has an antipsychotic effect (Smith, 1992). There are several other pathologic conditions in which DMSO serves as a treatment or as a solvent for delivering drugs. One study performed in guinea pigs papillary muscle suggest that DMSO, due to his hyperosmolarity, induces cell shrinkage; a 10% DMSO solution induce a cell shrinkage similar to 1.5 times hyperosmotic sucrose solution (Ogura et al., 1995). Various side effects of DMSO have also been reported, including chills, fever (Stroncek et al., 1991), severe hemolysis (Samoszuk et al., 1983), and gastrointestinal effects like nausea and vomiting (Davis et al., 1990).

While there are some reports that DMSO causes side effects like nausea and vomiting (Davis et al., 1990), rats treated with DMSO vehicle or Bay 73-6691 did not show signs of malaise in our conditioned taste aversion paradigm. However, in the CTA test, vehicle and Bay 73-6691 were given peripherally, and the vehicle only contained 20% DMSO. Thus, we can not exclude the possibility that animals, which received 75% DMSO centrally, could have suffered from malaise. It is possible that a different CTA paradigm, inducing a CTA after centrally administered Bay 73-6691, might produce a different outcome; malaise following DMSO or Bay 73-6691 would then perhaps have been detected. It is safe to say that DMSO is

often used as a solvent, but its side effects are commonly neglected. With its variable uses and effects, it is possible that DMSO also interfered in our experiments in an unknown way.

Even though our experiments with Bay 73-6691 beared some inherent risk because very little is known about the in-vivo effects of this drug, the lack of an amylin effect in many of our experiments was quite unforeseen. Interestingly, it has recently been reported by Woods and Langhans (Woods and Langhans, 2012) that effects observed following treatment with compounds that alter food intake can sometimes be difficult to reproduce from trial-to trial or from lab-to-lab. Woods recounts problems from his own laboratory relating to icv infusion of insulin; for many years, central insulin produced a stable anorectic effect, but at some point the effect was not reproducible. Regardless of whether the same original conditions were used or the conditions were optimized, the effect remained absent, until suddenly it appeared again. The reason why the anorectic effect of central insulin disappeared or reappeared remained unclear. Similar inconsistencies have been observed for the peptides that reduce food intake, including leptin and PYY, as well as for signals that are typically thought to increase food intake, such as mercaptoacetate and NPY (Woods and Langhans, 2012) What exactly happens mechanistically when a peptide or drug suddenly does not seem to reproduce well-described effects is often unclear. Similarly, in our experiments with peripheral administration of Bay 73-6691, we were unable to induce a clear anorectic effect of amylin, somehow confirming this finding of Woods. One explanation for this phenomenon might be a learning effect in the animals. Woods suggested that animals might learn to ignore drugs as a satiation factor when they are consistently stimulated with it under a specific situation. Therefore we can't exclude that the lack of amylin's anorectic effect and the weak or absent c-Fos expression, may have had its origin in some learning effect, which desensitized our animals against amylin.

6.2 The role of the AP in amylin-induced restoration of leptin sensitivity

The aim of the second study was to determine the role of the AP in the amylin-induced restoration of leptin sensitivity in DIO rats. The experiment's success heavily depended on our ability to replicate the findings of Roth and colleagues (Roth et al., 2008). Roth tested the impact of chronic amylin infusion (100 μg/kg/d; 14 days) alone and in combination with

leptin (500 μg/kg/d; 14 days) on body weight, food intake and the leptin-induced pSTAT3 signal in the brain of DIO-prone rats. Amylin treatment decreased food intake and bodyweight compared to vehicle; in combination with leptin an additional 16% reduction in food intake and an additional 4% weight loss was achieved. In the same study, leptin alone had no effect, because DIO-prone rats are leptin-resistant. These results suggested clearly that amylin restored leptin sensitivity to produce synergistic effects on body weight. Roth also tested if amylin pretreatment restored leptin-induced pSTAT3 signaling in the hypothalamus and the hindbrain. Indeed, amylin pretreatment for 7 days led to a 43% increase in leptin-induced pSTAT3 signaling in the VMH relative to vehicle-treated and pair-fed rats.

Unfortuntely, we were unable to replicate these findings in our cohort of DIO rats, making the contribution of the AP in our paradigm using AP-lesioned animals difficult to decipher. In other words, we did not observe any impact of amylin pretreatment on leptin's capacity to reduce food intake in an acute test in sham lesioned animals, nor any changes in leptin induced pSTAT3 signaling between the groups. All rats treated with terminal leptin mounted a pSTAT3 response, regardless of whether or not they were pretreated with amylin. While this indicated that rats were leptin sensitive, none of these rats showed a behavioral response to leptin. While we attempted to closely mimic the experimental design of Roth, some variations in our experiment possibly contributed to the disparate results. One difference in the experimental design was the DIO rat model. According to the methods established by Levin and colleagues (Levin et al., 1997) we genereated DIO rats by placing outbred Sprague Dawley rats on a high fat diet for two weeks, after which body weight and growth patterns were assessed. The tertile gaining the highest percentage of weight while on the high fat diet was designated DIO and used in our experiment. The rats were maintained on the high fat diet until the start of the chronic amylin treatment. The group of Roth used DIO-prone rats which are rats selectively bred over many generations for their propensity to become obese, even in the absence of a high fat diet. In fact, it is not clear from Roth's study if the DIO-prone rats were ever given access to a high fat diet, which on its own may have effects on neuronal leptin sensitivity, even during short term exposure (Olofsson et al., 2013). While the two DIO rat models exhibit many similarities, the extent of amylin and leptin to synergize in our DIO outbred model had not been formally established before or compared to that in DIO-prone rat.

Our study also had slight modifications in amylin and leptin delivery times, doses, and vehicles. In both studies amylin was administered subcutaneously (100 μg/kg/day) for up to 14 days; however in the Roth study, amylin was diluted in a vehicle of 50% DMSO, while we used saline as a vehicle. In both studies, chronic amylin treatment reduced body weight in intact DIO rats, however the effect of amylin appeared much stronger in Roth's study, suggesting that use of a DMSO vehicle may actually have enhanced the effectiveness of amylin, or possibly preserved its activity. Differences in leptin treatment may also have altered our results. Roth tested the effect of chronic co-administration of leptin and amylin on food intake, while we opted to test whether chronic amylin infusion modified the feeding response to acute leptin treatment in DIO rats. Unlike the Roth study, we observed no effect of chronic amylin treatment on acute leptin, suggesting that either the synergy between amylin and leptin is only displayed when both treatments are given chronically, or that the dose of the acute leptin treatment was not high enough to elicit an anorexic response. Indeed, Roth's group used a terminal dose of 15 mg/kg leptin to induce pSTAT3 in the brain, compared to 5 mg/kg of leptin in our study. Interestingly, in our experiment all animals that were treated with leptin showed a pSTAT 3 response, regardless of whether they were treated with amylin or not. This indicates that the dose difference might not matter, at least not concerning the induction of a strong pSTAT3 response.

The discrepancy between our results and those of Roth makes it difficult to conclude whether the AP is a critical brain center for the synergistic effects of amylin and leptin. We can confidently conclude that the area postrema is not required for leptin's induction of the pSTAT3 signal in the VMH. Further, we have some additional data showing that leptin-induced pSTAT3 in the NTS was also not altered in APX rats, again confirming the minor role of the AP in leptin signaling. The results of this experiment, unfortunately, do not shed new light on the mechanism underlying amylin and leptin's interaction; the AP can neither be confirmed nor excluded as a critical site of action. Given the difficulties we encountered when trying to replicate Roth's findings, it is clear that alternative strategies are necessary to test how amylin and leptin synergize to promote enhanced weight loss and reduction of food intake.

7 References

Adams, T.D., Davidson, L.E., Litwin, S.E., Kolotkin, R.L., LaMonte, M.J., Pendleton, R.C., Strong, M.B., Vinik, R., Wanner, N.A., Hopkins, P.N., Gress, R.E., Walker, J.M., Cloward, T.V., Nuttall, R.T., Hammoud, A., Greenwood, J.L., Crosby, R.D., McKinlay, R., Simper, S.C., Smith, S.C., Hunt, S.C., 2012, Health benefits of gastric bypass surgery after 6 years. Jama 308, 1122-1131.

Anand, B.K., Dua, S., 1955, Feeding responses induced by electrical stimulation of the hypothalamus in cat. Indian J Med Res 43, 113-122.

Andreeva, S.G., Dikkes, P., Epstein, P.M., Rosenberg, P.A., 2001, Expression of cGMP-specific phosphodiesterase 9A mRNA in the rat brain. J Neurosci 21, 9068-9076.

Banks, W.A., Farrell, C.L., 2003, Impaired transport of leptin across the blood-brain barrier in obesity is acquired and reversible. Am J Physiol Endocrinol Metab 285, E10-15.

Banks, W.A., Kastin, A.J., Maness, L.M., Huang, W., Jaspan, J.B., 1995, Permeability of the blood-brain barrier to amylin. Life Sci 57, 1993-2001.

Baskin, D.G., Breininger, J.F., Schwartz, M.W., 1999, Leptin receptor mRNA identifies a subpopulation of neuropeptide Y neurons activated by fasting in rat hypothalamus. Diabetes 48, 828-833.

Baumann, H., Morella, K.K., White, D.W., Dembski, M., Bailon, P.S., Kim, H., Lai, C.F., Tartaglia, L.A., 1996, The full-length leptin receptor has signaling capabilities of interleukin 6-type cytokine receptors. Proc Natl Acad Sci U S A 93, 8374-8378.

Beavo, J.A., 1995, Cyclic nucleotide phosphodiesterases: functional implications of multiple isoforms. Physiol Rev 75, 725-748.

Bentley, J.K., 2005, Immunoprecipitation of PDE2 phosphorylated and inactivated by an associated protein kinase. Methods Mol Biol 307, 211-223.

Blevins, J.E., Stanley, B.G., Reidelberger, R.D., 2000, Brain regions where cholecystokinin suppresses feeding in rats. Brain Res 860, 1-10.

Boolell, M., Allen, M.J., Ballard, S.A., Gepi-Attee, S., Muirhead, G.J., Naylor, A.M., Osterloh, I.H., Gingell, C., 1996, Sildenafil: an orally active type 5 cyclic GMP-specific phosphodiesterase inhibitor for the treatment of penile erectile dysfunction. Int J Impot Res 8, 47-52.

Broadwell, R.D., Salcman, M., Kaplan, R.S., 1982, Morphologic effect of dimethyl sulfoxide on the blood-brain barrier. Science 217, 164-166.

Broberger, C., Johansen, J., Johansson, C., Schalling, M., Hokfelt, T., 1998, The neuropeptide Y/agouti gene-related protein (AGRP) brain circuitry in normal, anorectic, and monosodium glutamate-treated mice. Proc Natl Acad Sci U S A 95, 15043-15048.

Buffa, R., Solcia, E., Go, V.L., 1976, Immunohistochemical identification of the cholecystokinin cell in the intestinal mucosa. Gastroenterology 70, 528-532.

Caro, J.F., Kolaczynski, J.W., Nyce, M.R., Ohannesian, J.P., Opentanova, I., Goldman, W.H., Lynn, R.B., Zhang, P.L., Sinha, M.K., Considine, R.V., 1996, Decreased cerebrospinal-fluid/serum leptin ratio in obesity: a possible mechanism for leptin resistance. Lancet 348, 159-161.

Cheung, W.Y., 1967, Properties of cyclic 3',5'-nucleotide phosphodiesterase from rat brain. Biochemistry 6, 1079-1087.

Cone, R.D., 2005, Anatomy and regulation of the central melanocortin system. Nat Neurosci 8, 571-578.
Considine, R.V., Sinha, M.K., Heiman, M.L., Kriauciunas, A., Stephens, T.W., Nyce, M.R., Ohannesian, J.P., Marco, C.C., McKee, L.J., Bauer, T.L., et al., 1996, Serum immunoreactive-leptin concentrations in normal-weight and obese humans. N Engl J Med 334, 292-295.
Cooper, G.J., Willis, A.C., Clark, A., Turner, R.C., Sim, R.B., Reid, K.B., 1987, Purification and characterization of a peptide from amyloid-rich pancreases of type 2 diabetic patients. Proc Natl Acad Sci U S A 84, 8628-8632.
Curran, T., Morgan, J.I., 1995, Fos: an immediate-early transcription factor in neurons. J Neurobiol 26, 403-412.
Davis, J.M., Rowley, S.D., Braine, H.G., Piantadosi, S., Santos, G.W., 1990, Clinical toxicity of cryopreserved bone marrow graft infusion. Blood 75, 781-786.
Deninno, M.P., Andrews, M., Bell, A.S., Chen, Y., Eller-Zarbo, C., Eshelby, N., Etienne, J.B., Moore, D.E., Palmer, M.J., Visser, M.S., Yu, L.J., Zavadoski, W.J., Michael Gibbs, E., 2009, The discovery of potent, selective, and orally bioavailable PDE9 inhibitors as potential hypoglycemic agents. Bioorg Med Chem Lett 19, 2537-2541.
Drenick, E.J., Bale, G.S., Seltzer, F., Johnson, D.G., 1980, Excessive mortality and causes of death in morbidly obese men. Jama 243, 443-445.
Edwards GL, G.B., Jodka C, Dilts RP, Miller CC, Young A, 1998, Area postrema (AP)-lesions block the regulation of gastric emptying by amylin. Neurogastroenterol Motil.
Elmquist, J.K., Bjorbaek, C., Ahima, R.S., Flier, J.S., Saper, C.B., 1998, Distributions of leptin receptor mRNA isoforms in the rat brain. J Comp Neurol 395, 535-547.
Fisher, D.A., Smith, J.F., Pillar, J.S., St Denis, S.H., Cheng, J.B., 1998, Isolation and characterization of PDE9A, a novel human cGMP-specific phosphodiesterase. J Biol Chem 273, 15559-15564.
Gedulin, B.R., Jodka, C.M., Herrmann, K., Young, A.A., 2006, Role of endogenous amylin in glucagon secretion and gastric emptying in rats demonstrated with the selective antagonist, AC187. Regul Pept 137, 121-127.
Ghilardi, N., Ziegler, S., Wiestner, A., Stoffel, R., Heim, M.H., Skoda, R.C., 1996, Defective STAT signaling by the leptin receptor in diabetic mice. Proc Natl Acad Sci U S A 93, 6231-6235.
Gibbs, J., Young, R.C., Smith, G.P., 1997, Cholecystokinin decreases food intake in rats. 1973. Obes Res 5, 284-290.
Guipponi, M., Scott, H.S., Kudoh, J., Kawasaki, K., Shibuya, K., Shintani, A., Asakawa, S., Chen, H., Lalioti, M.D., Rossier, C., Minoshima, S., Shimizu, N., Antonarakis, S.E., 1998, Identification and characterization of a novel cyclic nucleotide phosphodiesterase gene (PDE9A) that maps to 21q22.3: alternative splicing of mRNA transcripts, genomic structure and sequence. Hum Genet 103, 386-392.
Halaas, J.L., Gajiwala, K.S., Maffei, M., Cohen, S.L., Chait, B.T., Rabinowitz, D., Lallone, R.L., Burley, S.K., Friedman, J.M., 1995, Weight-reducing effects of the plasma protein encoded by the obese gene. Science 269, 543-546.
Haskell-Luevano, C., Chen, P., Li, C., Chang, K., Smith, M.S., Cameron, J.L., Cone, R.D., 1999, Characterization of the neuroanatomical distribution of agouti-related protein immunoreactivity in the rhesus monkey and the rat. Endocrinology 140, 1408-1415.
Hollander, P., Maggs, D.G., Ruggles, J.A., Fineman, M., Shen, L., Kolterman, O.G., Weyer, C., 2004, Effect of pramlintide on weight in overweight and obese insulin-treated type 2 diabetes patients. Obes Res 12, 661-668.

Howard, J.K., Cave, B.J., Oksanen, L.J., Tzameli, I., Bjorbaek, C., Flier, J.S., 2004, Enhanced leptin sensitivity and attenuation of diet-induced obesity in mice with haploinsufficiency of Socs3. Nat Med 10, 734-738.

Hubschle, T., Thom, E., Watson, A., Roth, J., Klaus, S., Meyerhof, W., 2001, Leptin-induced nuclear translocation of STAT3 immunoreactivity in hypothalamic nuclei involved in body weight regulation. J Neurosci 21, 2413-2424.

Ihle, J.N., Kerr, I.M., 1995, Jaks and Stats in signaling by the cytokine receptor superfamily. Trends Genet 11, 69-74.

Ikeda, Y., Long, D.M., 1990, Comparative effects of direct and indirect hydroxyl radical scavengers on traumatic brain oedema. Acta Neurochir Suppl (Wien) 51, 74-76.

Kelly, T., Yang, W., Chen, C.S., Reynolds, K., He, J., 2008, Global burden of obesity in 2005 and projections to 2030. Int J Obes (Lond) 32, 1431-1437.

Kleiman, R.J., Chapin, D.S., Christoffersen, C., Freeman, J., Fonseca, K.R., Geoghegan, K.F., Grimwood, S., Guanowsky, V., Hajos, M., Harms, J.F., Helal, C.J., Hoffmann, W.E., Kocan, G.P., Majchrzak, M.J., McGinnis, D., McLean, S., Menniti, F.S., Nelson, F., Roof, R., Schmidt, A.W., Seymour, P.A., Stephenson, D.T., Tingley, F.D., Vanase-Frawley, M., Verhoest, P.R., Schmidt, C.J., 2012, Phosphodiesterase 9A regulates central cGMP and modulates responses to cholinergic and monoaminergic perturbation in vivo. J Pharmacol Exp Ther 341, 396-409.

Knight, Z.A., Hannan, K.S., Greenberg, M.L., Friedman, J.M., 2010, Hyperleptinemia is required for the development of leptin resistance. PLoS One 5, e11376.

le Roux, C.W., Bueter, M., Theis, N., Werling, M., Ashrafian, H., Lowenstein, C., Athanasiou, T., Bloom, S.R., Spector, A.C., Olbers, T., Lutz, T.A., 2011, Gastric bypass reduces fat intake and preference. Am J Physiol Regul Integr Comp Physiol 301, R1057-1066.

Levin, B.E., Dunn-Meynell, A.A., Balkan, B., Keesey, R.E., 1997, Selective breeding for diet-induced obesity and resistance in Sprague-Dawley rats. Am J Physiol 273, R725-730.

Licinio, J., Caglayan, S., Ozata, M., Yildiz, B.O., de Miranda, P.B., O'Kirwan, F., Whitby, R., Liang, L., Cohen, P., Bhasin, S., Krauss, R.M., Veldhuis, J.D., Wagner, A.J., DePaoli, A.M., McCann, S.M., Wong, M.L., 2004, Phenotypic effects of leptin replacement on morbid obesity, diabetes mellitus, hypogonadism, and behavior in leptin-deficient adults. Proc Natl Acad Sci U S A 101, 4531-4536.

Liddle, R.A., Goldfine, I.D., Rosen, M.S., Taplitz, R.A., Williams, J.A., 1985, Cholecystokinin bioactivity in human plasma. Molecular forms, responses to feeding, and relationship to gallbladder contraction. J Clin Invest 75, 1144-1152.

Lin, S., Thomas, T.C., Storlien, L.H., Huang, X.F., 2000, Development of high fat diet-induced obesity and leptin resistance in C57Bl/6J mice. Int J Obes Relat Metab Disord 24, 639-646.

Lutz, T.A., Geary, N., Szabady, M.M., Del Prete, E., Scharrer, E., 1995, Amylin decreases meal size in rats. Physiol Behav 58, 1197-1202.

Lutz, T.A., Senn, M., Althaus, J., Del Prete, E., Ehrensperger, F., Scharrer, E., 1998, Lesion of the area postrema/nucleus of the solitary tract (AP/NTS) attenuates the anorectic effects of amylin and calcitonin gene-related peptide (CGRP) in rats. Peptides 19, 309-317.

Maffei, M., Halaas, J., Ravussin, E., Pratley, R.E., Lee, G.H., Zhang, Y., Fei, H., Kim, S., Lallone, R., Ranganathan, S., et al., 1995, Leptin levels in human and rodent: measurement of plasma leptin and ob RNA in obese and weight-reduced subjects. Nat Med 1, 1155-1161.

Malnick, S.D., Knobler, H., 2006, The medical complications of obesity. Qjm 99, 565-579.
Matsuoka, I., Giuili, G., Poyard, M., Stengel, D., Parma, J., Guellaen, G., Hanoune, J., 1992, Localization of adenylyl and guanylyl cyclase in rat brain by in situ hybridization: comparison with calmodulin mRNA distribution. J Neurosci 12, 3350-3360.
McGlone, J.J., Ritter, S., Kelley, K.W., 1980, The antiaggressive effect of lithium is abolished by area postrema lesion. Physiol Behav 24, 1095-1100.
Michel, S., Becskei, C., Erguven, E., Lutz, T.A., Riediger, T., 2007, Diet-derived nutrients modulate the effects of amylin on c-Fos expression in the area postrema and on food intake. Neuroendocrinology 86, 124-135.
Mollet, A., Gilg, S., Riediger, T., Lutz, T.A., 2004, Infusion of the amylin antagonist AC 187 into the area postrema increases food intake in rats. Physiol Behav 81, 149-155.
Morley, J.E., Flood, J.F., 1991, Amylin decreases food intake in mice. Peptides 12, 865-869.
Morley, J.E., Suarez, M.D., Mattamal, M., Flood, J.F., 1997, Amylin and food intake in mice: effects on motivation to eat and mechanism of action. Pharmacol Biochem Behav 56, 123-129.
Muff, R., Buhlmann, N., Fischer, J.A., Born, W., 1999, An amylin receptor is revealed following co-transfection of a calcitonin receptor with receptor activity modifying proteins-1 or -3. Endocrinology 140, 2924-2927.
Munzberg, H., Flier, J.S., Bjorbaek, C., 2004, Region-specific leptin resistance within the hypothalamus of diet-induced obese mice. Endocrinology 145, 4880-4889.
Nachman, M., Ashe, J.H., 1973, Learned taste aversions in rats as a function of dosage, concentration, and route of administration of LiCl. Physiol Behav 10, 73-78.
Nakagawa, T., Ukai, K., Ohyama, T., Gomita, Y., Okamura, H., 2000, Effects of chronic administration of sibutramine on body weight, food intake and motor activity in neonatally monosodium glutamate-treated obese female rats: relationship of antiobesity effect with monoamines. Exp Anim 49, 239-249.
Ogawa, A., Harris, V., McCorkle, S.K., Unger, R.H., Luskey, K.L., 1990, Amylin secretion from the rat pancreas and its selective loss after streptozotocin treatment. J Clin Invest 85, 973-976.
Ogura, T., Shuba, L.M., McDonald, T.F., 1995, Action potentials, ionic currents and cell water in guinea pig ventricular preparations exposed to dimethyl sulfoxide. J Pharmacol Exp Ther 273, 1273-1286.
Olofsson, L.E., Unger, E.K., Cheung, C.C., Xu, A.W., 2013, Modulation of AgRP-neuronal function by SOCS3 as an initiating event in diet-induced hypothalamic leptin resistance. Proc Natl Acad Sci U S A 110, E697-706.
Parkin, J., Shea, C., Sant, G.R., 1997, Intravesical dimethyl sulfoxide (DMSO) for interstitial cystitis--a practical approach. Urology 49, 105-107.
Paxinos, G., Watson, C., 1998, The rat brain in stereotaxic coordinates, 4th Edition. Academic Press, San Diego.
Potes, C.S., Boyle, C.N., Wookey, P.J., Riediger, T., Lutz, T.A., 2012, Involvement of the extracellular signal-regulated kinase 1/2 signaling pathway in amylin's eating inhibitory effect. Am J Physiol Regul Integr Comp Physiol 302, R340-351.
Rajfer, J., Aronson, W.J., Bush, P.A., Dorey, F.J., Ignarro, L.J., 1992, Nitric oxide as a mediator of relaxation of the corpus cavernosum in response to nonadrenergic, noncholinergic neurotransmission. N Engl J Med 326, 90-94.
Reidelberger, R.D., Arnelo, U., Granqvist, L., Permert, J., 2001, Comparative effects of amylin and cholecystokinin on food intake and gastric emptying in rats. Am J Physiol Regul Integr Comp Physiol 280, R605-611.

Rentero, C., Monfort, A., Puigdomenech, P., 2003, Identification and distribution of different mRNA variants produced by differential splicing in the human phosphodiesterase 9A gene. Biochem Biophys Res Commun 301, 686-692.

Riediger, T., Schmid, H.A., Lutz, T., Simon, E., 2001, Amylin potently activates AP neurons possibly via formation of the excitatory second messenger cGMP. Am J Physiol Regul Integr Comp Physiol 281, R1833-1843.

Riediger, T., Zuend, D., Becskei, C., Lutz, T.A., 2004, The anorectic hormone amylin contributes to feeding-related changes of neuronal activity in key structures of the gut-brain axis. Am J Physiol Regul Integr Comp Physiol 286, R114-122.

Roth, J.D., Roland, B.L., Cole, R.L., Trevaskis, J.L., Weyer, C., Koda, J.E., Anderson, C.M., Parkes, D.G., Baron, A.D., 2008, Leptin responsiveness restored by amylin agonism in diet-induced obesity: evidence from nonclinical and clinical studies. Proc Natl Acad Sci U S A 105, 7257-7262.

Roth, J.D., Trevaskis, J.L., Turek, V.F., Parkes, D.G., 2010, "Weighing in" on synergy: preclinical research on neurohormonal anti-obesity combinations. Brain Res 1350, 86-94.

Rowland, N.E., Crews, E.C., Gentry, R.M., 1997, Comparison of Fos induced in rat brain by GLP-1 and amylin. Regul Pept 71, 171-174.

Samoszuk, M., Reid, M.E., Toy, P.T., 1983, Intravenous dimethylsulfoxide therapy causes severe hemolysis mimicking a hemolytic transfusion reaction. Transfusion 23, 405.

Schmidt, H.H., Lohmann, S.M., Walter, U., 1993, The nitric oxide and cGMP signal transduction system: regulation and mechanism of action. Biochim Biophys Acta 1178, 153-175.

Sexton, P.M., Paxinos, G., Kenney, M.A., Wookey, P.J., Beaumont, K., 1994, In vitro autoradiographic localization of amylin binding sites in rat brain. Neuroscience 62, 553-567.

Shah, N.R., Braverman, E.R., 2012, Measuring adiposity in patients: the utility of body mass index (BMI), percent body fat, and leptin. PLoS One 7, e33308.

Shirley, S.W., Stewart, B.H., Mirelman, S., 1978, Dimethyl sulfoxide in treatment of inflammatory genitourinary disorders. Urology 11, 215-220.

Smith, R.S., 1992, A comprehensive macrophage-T-lymphocyte theory of schizophrenia. Med Hypotheses 39, 248-257.

Smith, S.R., Blundell, J.E., Burns, C., Ellero, C., Schroeder, B.E., Kesty, N.C., Chen, K.S., Halseth, A.E., Lush, C.W., Weyer, C., 2007, Pramlintide treatment reduces 24-h caloric intake and meal sizes and improves control of eating in obese subjects: a 6-wk translational research study. Am J Physiol Endocrinol Metab 293, E620-627.

Stricker, E.M., Curtis, K.S., Peacock, K.A., Smith, J.C., 1997, Rats with area postrema lesions have lengthy eating and drinking bouts when fed ad libitum: implications for feedback inhibition of ingestive behavior. Behav Neurosci 111, 623-632.

Stroncek, D.F., Fautsch, S.K., Lasky, L.C., Hurd, D.D., Ramsay, N.K., McCullough, J., 1991, Adverse reactions in patients transfused with cryopreserved marrow. Transfusion 31, 521-526.

Tartaglia, L.A., 1997, The leptin receptor. J Biol Chem 272, 6093-6096.

Tartaglia, L.A., Dembski, M., Weng, X., Deng, N., Culpepper, J., Devos, R., Richards, G.J., Campfield, L.A., Clark, F.T., Deeds, J., Muir, C., Sanker, S., Moriarty, A., Moore, K.J., Smutko, J.S., Mays, G.G., Wool, E.A., Monroe, C.A., Tepper, R.I., 1995, Identification and expression cloning of a leptin receptor, OB-R. Cell 83, 1263-1271.

Trevaskis, J.L., Coffey, T., Cole, R., Lei, C., Wittmer, C., Walsh, B., Weyer, C., Koda, J., Baron, A.D., Parkes, D.G., Roth, J.D., 2008, Amylin-mediated restoration of leptin responsiveness in diet-induced obesity: magnitude and mechanisms. Endocrinology 149, 5679-5687.

Trevaskis, J.L., Lei, C., Koda, J.E., Weyer, C., Parkes, D.G., Roth, J.D., 2010, Interaction of leptin and amylin in the long-term maintenance of weight loss in diet-induced obese rats. Obesity (Silver Spring) 18, 21-26.

Turek, V.F., Trevaskis, J.L., Levin, B.E., Dunn-Meynell, A.A., Irani, B., Gu, G., Wittmer, C., Griffin, P.S., Vu, C., Parkes, D.G., Roth, J.D., 2010, Mechanisms of amylin/leptin synergy in rodent models. Endocrinology 151, 143-152.

van der Staay, F.J., Rutten, K., Barfacker, L., Devry, J., Erb, C., Heckroth, H., Karthaus, D., Tersteegen, A., van Kampen, M., Blokland, A., Prickaerts, J., Reymann, K.G., Schroder, U.H., Hendrix, M., 2008, The novel selective PDE9 inhibitor BAY 73-6691 improves learning and memory in rodents. Neuropharmacology 55, 908-918.

WHO 2013. Obesity and Overweight; Fact sheet No.311.

Widdowson, P.S., Upton, R., Buckingham, R., Arch, J., Williams, G., 1997, Inhibition of food response to intracerebroventricular injection of leptin is attenuated in rats with diet-induced obesity. Diabetes 46, 1782-1785.

Woods, S.C., Langhans, W., 2012, Inconsistencies in the assessment of food intake. Am J Physiol Endocrinol Metab 303, E1408-1418.

Woods, S.C., Seeley, R.J., 2000, Adiposity signals and the control of energy homeostasis. Nutrition 16, 894-902.

Woods, S.C., Seeley, R.J., Porte, D., Jr., Schwartz, M.W., 1998, Signals that regulate food intake and energy homeostasis. Science 280, 1378-1383.

Wunder, F., Tersteegen, A., Rebmann, A., Erb, C., Fahrig, T., Hendrix, M., 2005, Characterization of the first potent and selective PDE9 inhibitor using a cGMP reporter cell line. Mol Pharmacol 68, 1775-1781.

Young, A., 1997, Role of amylin in nutrient intake - animal studies. Diabet Med 14 Suppl 2, S14-18.

Young, A.A., Gedulin, B.R., Rink, T.J., 1996, Dose-responses for the slowing of gastric emptying in a rodent model by glucagon-like peptide (7-36) NH2, amylin, cholecystokinin, and other possible regulators of nutrient uptake. Metabolism 45, 1-3.

Zhang, Y., Proenca, R., Maffei, M., Barone, M., Leopold, L., Friedman, J.M., 1994, Positional cloning of the mouse obese gene and its human homologue. Nature 372, 425-432.

Zhang, Y., Scarpace, P.J., 2006, The role of leptin in leptin resistance and obesity. Physiol Behav 88, 249-256.

Zorrilla, E.P., Inoue, K., Fekete, E.M., Tabarin, A., Valdez, G.R., Koob, G.F., 2005, Measuring meals: structure of prandial food and water intake of rats. Am J Physiol Regul Integr Comp Physiol 288, R1450-1467.

Zuger, D., Forster, K., Lutz, T.A., Riediger, T., 2013, Amylin and GLP-1 target different populations of area postrema neurons that are both modulated by nutrient stimuli. Physiol Behav 112-113C, 61-69.

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